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The Transcription Factor NIGT1.2 Modulates Both Phosphate Uptake and Nitrate Influx during Phosphate Starvation in Arabidopsis and Maize

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Phosphorus and nitrogen are essential macronutrients for plant growth and crop production. During phosphate (Pi) starvation, plants enhanced Pi but reduced nitrate (NO_3^-) uptake capacity, and the mechanism is unclear. Here, we show that a GARP-type transcription factor NITRATE-INDUCIBLE, GARP-TYPE TRANSCRIPTIOANL REPRESSOR1.2 (NIGT1.2) coordinately modulates Pi and NO_3^- uptake in response to Pi starvation. Overexpression of *NIGT1.2* increased Pi uptake capacity but decreased NO_3^- uptake capacity in Arabidopsis (*Arabidopsis thaliana*). Furthermore, the *nigt1.1 nigt1.2* double mutant displayed reduced Pi uptake but enhanced NO_3^- uptake under low-Pi stress. During Pi starvation, NIGT1.2 directly up-regulated the transcription of the Pi transporter genes *PHOSPHATE TRANSPORTER1;1* (*PHT1;1*) and *PHOSPHATE TRANSPORTER1;4* (*PHT1;4*) and down-regulated expression of NO_3^- transporter gene *NITRATE TRANSPORTER1.1* (*NRT1.1*) by binding to *cis*-elements in their promoters. Further genetic assays demonstrated that *PHT1;1*, *PHT1;4*, and *NRT1.1* were genetically epistatic to *NIGT1.2*. We also identified similar regulatory pathway in maize (*Zea mays*). These data demonstrate that the transcription factor NIGT1.2 plays a central role in modulating low-Pi-dependent uptake of Pi and NO_3^-, tending toward maintenance of the phosphorus to nitrogen balance in plants during Pi starvation.

Introduction

Phosphorus (P) is a macronutrient that is essential for plant growth and crop production and is an important component of the fertilizers used to sustain modern agriculture. Approximately 50 million tons of P fertilizer is required annually for crop production worldwide, but crops assimilate no more than 30% of P fertilizer (Good and Beatty, 2011; López-Arredondo et al., 2014). Phosphate (Pi) is the least available nutrient in fertilizer because it is highly immobile in soil and easily bound to oxides and hydroxides of Fe^{3+} and Al^{3+} or converted to organic matter by microorganisms (Marschner and Rimmington, 1988; Raghothama, 1999; López-Arredondo et al., 2014). As a result, ~70% of cultivated land worldwide is deficient in plant-available Pi (Hinsinger, 2001; Kirkby and Johnston, 2008; López-Arredondo et al., 2014; Nguyen et al., 2015).

To maintain their growth under low-Pi stress, plants have evolved various strategies that overcome limited Pi availability. During Pi starvation, plants increase Pi uptake through alteration of root architecture and function (Péret et al., 2011; Liang et al., 2014; López-Arredondo et al., 2014), increases in phosphatase activity (López-Arredondo et al., 2014), and secretion of organic acids (Liang et al., 2014; López-Arredondo et al., 2014).

Nitrogen (N) is another macronutrient essential for plant growth, and nitrate in aerobic soils is a major N source for plants (Liu et al., 2017). Pi and NO_3^- acquisition in plants are interacting processes (Kant et al., 2011). Transcriptional profiling of Arabidopsis (*Arabidopsis thaliana*) and maize (*Zea mays*) have shown that P-deprived plants undergo substantial changes in the expression of many genes involved in nitrogenmetabolism, reduction, and uptake (Wu et al., 2003; Morcuende et al., 2007; Schlüter et al., 2013). During Pi starvation, NO_3^- uptake is reduced in various plant species, such as tobacco (*Nicotiana tabacum*; Rufty et al., 1990), barley (*Hordeum vulgare*; Rufty et al., 1991), soybean (*Glycine max*; Rufty et al., 1993), maize (de Magalhaes et al., 1998), bean (*Phaseolus vulgaris*; Gniazdowska et al., 1999), tomato (*Solanum lycopersicum*; de Groot et al., 2003), and lupin (*Lupinus luteus*; Kleinert et al., 2014). Pi starvation also decreases the activity of nitrate reductase in bean (*Phaseolus vulgaris*; Gniazdowska and Rychter, 2000) and N fixation in *Sesbania rostrata* (Aono et al., 2001) and lupin (Kleinert et al., 2014). However, the interaction and balance between NO_3^- and Pi in plants during Pi starvation has not been well studied.

Arabidopsis NITRATE-INDUCIBLE, GARP-TYPE TRANSCRIPTIONAL REPRESSOR1.2 (NIGT1.2), also named HYPERSENSITIVITY TO LOW PHOSPHATE-ELICITED PRIMARY ROOT SHORTENING1 HOMOLOG2 (HHO2), is a myb-related transcription factor and is a homolog to NIGT1 primitively identified in rice (*Oryza sativa*; Sawaki et al., 2013). Arabidopsis NIGT1.2/HHO2 can modulate the growth of primary and lateral roots in response to Pi starvation (Nagarajan et al., 2016). Recently, four NIGT1s have been reported to be negative regulators in the Arabidopsis response to nitrogen starvation and directly repress transcription of

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Background: Phosphorus (P) and nitrogen (N) are essential for all plants, and are often the elements that limit primary productivity. P mobility in soil is limited. Whereas soil concentrations of inorganic P, mainly in the form of orthophosphate (PO_4^{3-}; Pi), are low, those of organic P tend to be higher. As organic P cannot be absorbed by plants, the low Pi concentration in the soil results in low Pi availability for plant growth and survival. Nitrate in aerobic soils is a major N source for plants. During Pi starvation, plants enhance Pi uptake but reduce nitrate uptake; however, the underlying mechanism is unclear.

Question: What is the molecular mechanism by which plants modulate phosphate and nitrate uptake under Pi-deficient conditions?

Findings: We showed that under Pi-deficient and nitrate-sufficient conditions, the *Arabidopsis thaliana* transcription factors NIGT1.1 and NIGT1.2 had a dual role both as direct activators of Pi transporters and as direct repressors of nitrate transporters, and thereby balanced N and P uptake. A similar regulatory pathway was identified in maize.

Next steps: We established that NIGT1.1/1.2 modulates nitrate and phosphate uptake in response to the combined availability of P and N. Under various P/N combinations, NIGT1.1/1.2 may be modulated at both the transcriptional and posttranscriptional levels. We plan to investigate the mechanisms that regulate NIGT1 proteins under various P/N supply conditions.

high-affinity NO_3^- transporter genes *NITRATE TRANSPORTER2.1* (*NRT2.1*) and *NITRATE TRANSPORTER2.4* (*NRT2.4*; Kiba et al., 2018; Maeda et al., 2018). The *NIGT1* genes are transcriptionally regulated by the transcription factor PHOSPHATE STARVATION RESPONSE 1 (PHR1; Maeda et al., 2018), a master regulator of the Arabidopsis Pi-starvation response (Bustos et al., 2010).

In this study, we established that Arabidopsis NIGT1.2 coordinately modulated Pi and NO_3^- uptake during Pi starvation. The transcription of *NIGT1.2* was induced by low-Pi stress and NO_3^- in Arabidopsis. During Pi starvation, Arabidopsis NIGT1.2 bound to the promoters of the Pi transporter genes *PHT1;1* and *PHT1;4*, which increased Pi uptake, and also to the promoter of the NO_3^- transporter gene *NRT1.1*, which repressed NO_3^- acquisition. Pi deficiency also reduced NO_3^- uptake capacity in maize, where ZmNIGT1.2, the maize homolog of AtNIGT1.2, modulated low-Pi-dependent NO_3^- acquisition by down-regulating the maize NO_3^- transporter gene *ZmNPF2*. These findings reveal that Pi deficiency results in the antagonistic acquisition of Pi and NO_3^- through a process modulated by the transcription factor NIGT1.2.

Results

Pi Deficiency Enhances Pi and Represses NO_3^- Uptake in Arabidopsis

The Pi concentration in the soil solution is typically 10 μM or less, and plants often suffer Pi deficiency (Raghothama, 1999). A previous report showed that Pi and NO_3^- had an antagonistic interaction (Kant et al., 2011), and we wondered whether there was an interaction between Pi and NO_3^- uptake under low-Pi stress. When 7-d-old Arabidopsis seedlings were transferred to hydroponic medium with (+P) or without (−P) Pi for 14 d, the growth of Arabidopsis was impaired by Pi deficiency (Figure 1A), and the Pi uptake rate was significantly enhanced during Pi starvation (Figure 1B), similar to previous reports (Devaiah et al., 2007). Measurement of N-related physiological parameters showed that N concentration was lower in Arabidopsis seedlings grown under Pi-deficient conditions than in those grown under Pi-sufficient conditions (Figure 1C) and that Pi deficiency reduced Arabidopsis nitrate concentration (Figure 1D) and nitrate uptake (Figure 1E).

The molecular mechanism of NO_3^- uptake in Arabidopsis is well characterized (Wang et al., 2012, 2018). Arabidopsis has two NO_3^- uptake systems: a high-affinity system and a low-affinity system. Two NO_3^- transporters, NRT1.2 (a low-affinity NO_3^- transporter) and NRT1.1 (a dual-affinity NO_3^- transporter), are involved in NO_3^- uptake under nitrate-sufficient conditions (Tsay et al., 1993; Huang et al., 1999; Liu et al., 1999; Wang et al., 2012). The NRT2 family members, such as NRT2.1 and NRT2.4, participate in high-affinity NO_3^- uptake (Wang et al., 2018). Measuring the transcripts of *NRT1s* and *NRT2s* under low-Pi stress indicated that *NRT1.1* transcription was significantly repressed by low-Pi stress, whereas the transcripts of *NRT1.2*, *NRT2.1*, and *NRT2.4* were not modulated by low-Pi stress (Figure 1F), suggesting that Pi deficiency represses Arabidopsis NO_3^- uptake via down-regulation of *NRT1.1*.

NIGT1.2 Positively Modulates Pi Uptake during Pi Starvation

A previous report demonstrated that Arabidopsis PHT1;1 and PHT1;4 are the main Pi transporters participating in Pi uptake in roots (Shin et al., 2004). During Pi starvation, the transcription of *PHT1;1* and *PHT1;4* was elevated (Shin et al., 2004), and the expression of *NRT1.1* was repressed (Figure 1F). To screen for the transcription factor that directly modulates expression of *PHT1;1* and/or *NRT1.1*, we conducted a yeast one-hybrid assay using the promoters of *PHT1;1* and *NRT1.1* with a high-throughput Arabidopsis transcription factor screening system (Ou et al., 2011). At1g68670, also named *HHO2* (Nagarajan et al., 2016) or *NIGT1.2* (Kiba et al., 2018; Maeda et al., 2018), was isolated using both the *PHT1;1* promoter and *NRT1.1* promoter, suggesting that NIGT1.2 directly modulates the expression of *PHT1;1* and *NRT1.1*. As a transcription factor, the NIGT1.2 protein was exclusively localized

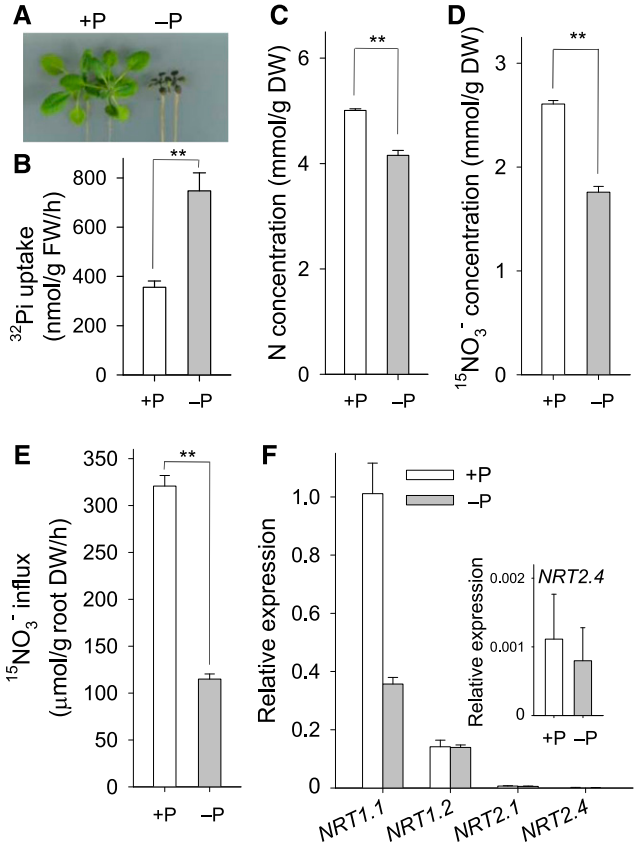

Figure 1. Pi Deficiency Enhances Arabidopsis Phosphate Uptake and Represses Nitrate Uptake.

(A) Phenotypic comparison of Arabidopsis wild-type plants grown in hydroponic solution with (+P) or without (−P) Pi for 14 d.

(B) Pi (^{32}P) uptake capacity was measured in 7-d-old Arabidopsis plants transferred to +P or −P hydroponic solution for 3 d. Data are shown as mean ± SE (n = 3).

(C) N concentration of 7-d-old Arabidopsis transferred to +P or −P hydroponic solution for 14 d. Data are shown as mean ± SE (n = 3).

(D) Nitrate concentration of 7-d-old Arabidopsis transferred to +P or −P hydroponic solution with 5 mM NO_3^- (5 atom% ^{15}N) for 14 d. Data are shown as mean ± SE (n = 3).

(E) Nitrate influx was measured in 7-d-old Arabidopsis transferred to MS (+P, 1.25 mM Pi) or low-Pi (−P, 10 μM Pi) medium for another 7 d. Data are shown as mean ± SE (n = 4).

(F) RT-qPCR analysis of *NRT1s* and *NRT2s* during Pi starvation. Seven-day-old wild-type Arabidopsis seedlings were transferred to MS medium (+P, 1.25 mM Pi) or low-Pi (−P, 10 μM Pi) medium for 5 d, and then roots were harvested at the indicated time points for RNA extraction. Data are shown as mean ± SE (n = 3). Asterisks in **(B)**, **(C)**, **(D)**, and **(E)** indicate significant differences compared with wild-type plants (#) by Student's t test: *P < 0.05; **P < 0.01.

in the nucleus (Supplemental Figure 1A; Kiba et al., 2018). Transcription of *NIGT1.2* was induced by Pi deficiency and NO_3^- treatment (Supplemental Figure 1B), similar to a previous reportby Kiba et al. (2018). A β-glucuronidase (GUS) staining assay further confirmed that the transcription of *NIGT1.2* was elevated under low-Pi stress (Figure 2A).

Given that *NIGT1.2* was induced during Pi starvation (Figures 2A; Supplemental Figure 1B; Kiba et al., 2018; Maeda et al., 2018), we decided to generate *NIGT1.2*-overexpressing Arabidopsis lines for further assessment. Two *NIGT1.2*-overexpressing lines, *35S:NIGT1.2-8* and *35S:NIGT1.2-23*, were selected for further study, because they showed gradated increases in *NIGT1.2* expression (Figure 2B). Seedlings from both lines showed higher Pi concentration than wild-type seedlings, consistent with their *NIGT1.2* expression levels (Figure 2C), suggesting that overexpression of *NIGT1.2* increased Arabidopsis Pi concentration. Next, we analyzed the Pi uptake rate further by transferring 10-d-old seedlings into a Pi uptake solution containing 500 μM Pi supplemented with ^{32}P orthophosphate and measuring the Pi uptake over a 6-h period. The *NIGT1.2*-overexpressing lines displayed a substantial increase in Pi uptake capacity compared with wild-type plants, and the increment was closely related to the *NIGT1.2* expression level (Figure 2D). These data indicate that overexpression of *NIGT1.2* enhances the Pi absorption in Arabidopsis.

To further confirm the function of NIGT1.2, we obtained three T-DNA insertion mutants of *NIGT1.2* from the Arabidopsis Biological Resource Center (http://abrc.osu.edu), which we named *nigt1.2-1*, *nigt1.2-2*, and *nigt1.2-3* (Supplemental Figure 2A). RT-qPCR analysis showed that transcription of *NIGT1.2* was knocked down in three *nigt1.2* mutants (Supplemental Figure 2B), whereas the Pi concentrations of the *nigt1.2* mutants were similar to that of wild-type plants under both Pi-sufficient and Pi-deficient conditions (Supplemental Figure 2C).

The *NIGT1.1/HHO3* was a homolog of *NIGT1.2* in the Arabidopsis genome (Medici et al., 2015; Maeda et al., 2018), and the *NIGT1.1* was also induced by low-Pi stress and NO_3^- (Supplemental Figure 3; Kiba et al., 2018; Maeda et al., 2018). We generated a *nigt1.1* mutant and *nigt1.1 nigt1.2* double mutant using CRISPR/Cas9 technology (Supplemental Figure 4). The *nigt1.1* mutant displayed a similar phenotype as the *nigt1.2* mutant and wild-type plants under low-Pi stress (Figure 2E) and showed a Pi concentration and Pi uptake rate similar to those of wild-type plants (Figures 2F and 2G). Interestingly, the *nigt1.1 nigt1.2* double mutants displayed a low-Pi sensitive phenotype and significantly reduced Pi concentrations and Pi uptake capacity compared with wild-type plants under Pi-deficient conditions, and there were no obvious differences in Pi concentration or uptake between *nigt1.1 nigt1.2* double mutants and wild-type plants when grown under Pi-sufficient conditions (Figures 2F and 2G). These data demonstrate that NIGT1.2 positively modulates Pi uptake during Pi starvation.

NIGT1.2 Directly Up-Regulates Pi Transporter Genes during Pi Starvation

NIGT1.2 modulated Pi uptake in response to environmental Pi supply (Figure 2), and the PHT1;1 and PHT1;4 are the main transporters for Pi uptake in roots in both low- and high-Pi environments (Shin et al., 2004). We hypothesized that NIGT1.2 modulates Pi uptake by directly regulating expression of *PHT1;1* and/or *PHT1;4*. RT-qPCR analysis showed that the transcription of both *PHT1;1* and *PHT1;4* was increased in the *NIGT1.2*-overexpressing lines, and the increments in the levels of *PHT1;1*

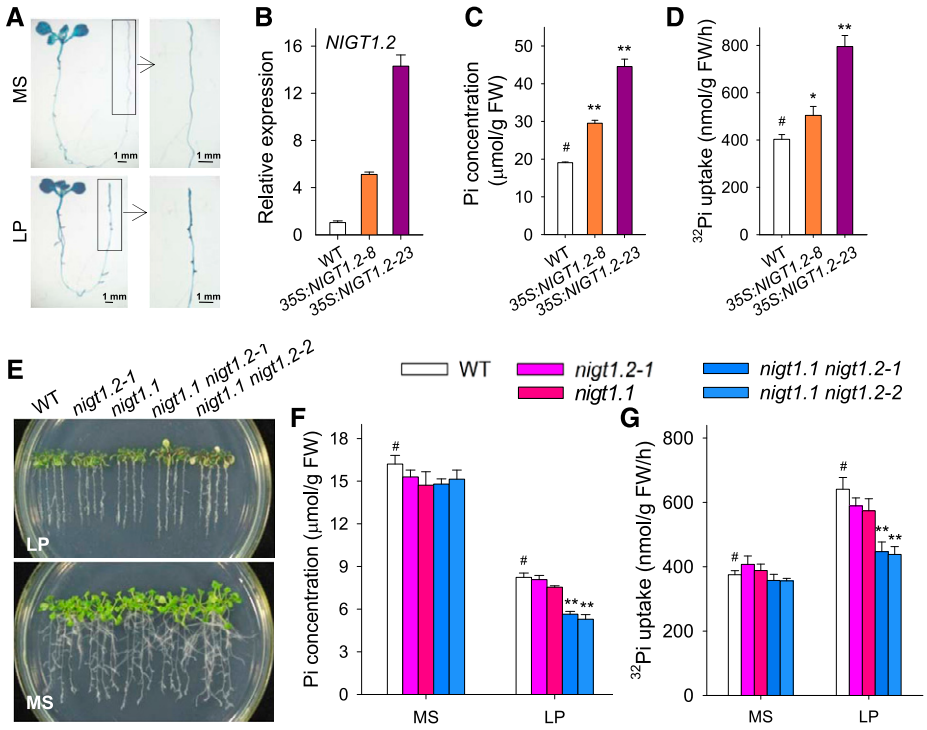

Figure 2. NIGT1.2 Positively Modulates Pi Acquisition in Arabidopsis during Pi Starvation.

(A) GUS staining assay of the *ProNIGT1.2:GUS* transgenic line. Seven-day-old *ProNIGT1.2:GUS* seedlings were transferred to MS or low-Pi (LP) medium for 5 d and then stained for GUS.

(B) Analysis of *NIGT1.2* expression using RT-qPCR in 10-d-old *NIGT1.2*-overexpressing and wild-type plants. Data are shown as mean ± SE ($n = 3$).

(C) Measurement of Pi concentration. Seven-day-old seedlings were transferred to MS medium for 5 d and then harvested for Pi concentration measurement. Data are shown as mean ± SE ($n = 3$).

(D) ^{32}Pi uptake capacity was measured in 10-d-old seedlings germinated and grown on MS medium. Data are shown as mean ± SE ($n = 3$).

(E) Phenotypic comparison of the *nigt1.2*, *nigt1.1*, *nigt1.1 nigt1.2* double mutants, and wild-type seedlings during Pi starvation. Seven-day-old seedlings were transferred to LP or MS medium for another 7 d, and then photographs were taken.

(F) Pi concentration measurement in 7-d-old seedlings grown on MS or LP medium for 5 d. Data are shown as mean ± SE ($n = 4$).

(G) ^{32}Pi uptake capacity was measured in 7-d-old seedlings grown on MS or LP medium for 3 d. Data are shown as mean ± SE ($n = 4$). Asterisks in **(C)**, **(D)**, **(F)**, and **(G)** indicate significant differences compared with wild-type plants (#) by Student's *t* test: *P < 0.05; **P < 0.01.

and *PHT1;4* were consistent with the *NIGT1.2* expression levels in *NIGT1.2*-overexpressing lines (Figure 3A). In addition, when we crossed the *ProPHT1;1:GUS* transgenic line (Wang et al., 2014) with the wild-type plants and *NIGT1.2*-overexpressing line (*35S:NIGT1.2-23*), GUS staining was enhanced in the *NIGT1.2*-overexpressing line (Figure 3B), indicating that NIGT1.2 positively regulated transcription of *PHT1;1*. We also tested the expression of *PHT1;1* and *PHT1;4* in the *nigt1.1 nigt1.2* double mutant and found that under Pi-sufficient conditions, there was no detectable difference between the *nigt1.1 nigt1.2* double mutant and the wild-type plants. In contrast, under low-Pi stress, transcription of both *PHT1;1* and *PHT1;4* was significantly enhanced, and these low-Pi-dependent enhancements were repressed in the *nigt1.1 nigt1.2* double mutant relative to wild-type plants (Figures 3C and 3D), suggesting that NIGT1.2 positively modulates *PHT1;1* and *PHT1;4* transcription under low-Pi stress.

Previous reports indicate that the transcription factor HYPERSENSITIVITY TO LOW PI-ELICITED PRIMARY ROOT SHORTENING1 (HRS1), a homolog of NIGT1.2, can bind to the up-regulatory elements AGANNNAAA and AAACNNAACC (Medici et al., 2015). The *cis*-element analysis showed that the promoters of *PHT1;1* and *PHT1;4* contained two (AGANNNAAA and AAACNNAACC) and one (AGANNNAAA) of the up-regulatory *cis*-motifs, respectively (Figure 3E). We next conducted a chromatin immunoprecipitation (ChIP) assay to confirm that NIGT1.2 bound to the *PHT1;1* and *PHT1;4* promoters in plants. Seven-day-old wild-type seedlings were transferred to Murashige and Skoog (MS) or low Pi (LP, with 10 μM Pi) medium for 5 d, and then the roots were harvested for ChIP assay using anti-NIGT1.2 antibody (Figure 3F). Chromatin immunoprecipitated with anti-NIGT1.2 antibody was mainly enriched in the P2 fragment of the *PHT1;1* promoter in plants grown under low-Pi stress, and no NIGT1.2 enrichment at either P1 or P2 of the *PHT1;1* promoter was detected under Pi-sufficient conditions (Figure 3G). NIGT1.2 also bound to the P1 fragment of the *PHT1;4* promoter during Pi starvation, but it did not bind to the *PHT1;4* promoter at all under Pi-sufficient conditions (Figure 3H). These data indicate that NIGT1.2 bound to the promoters of *PHT1;1* and *PHT1;4* under Pi-deficient conditions, which is consistent with the

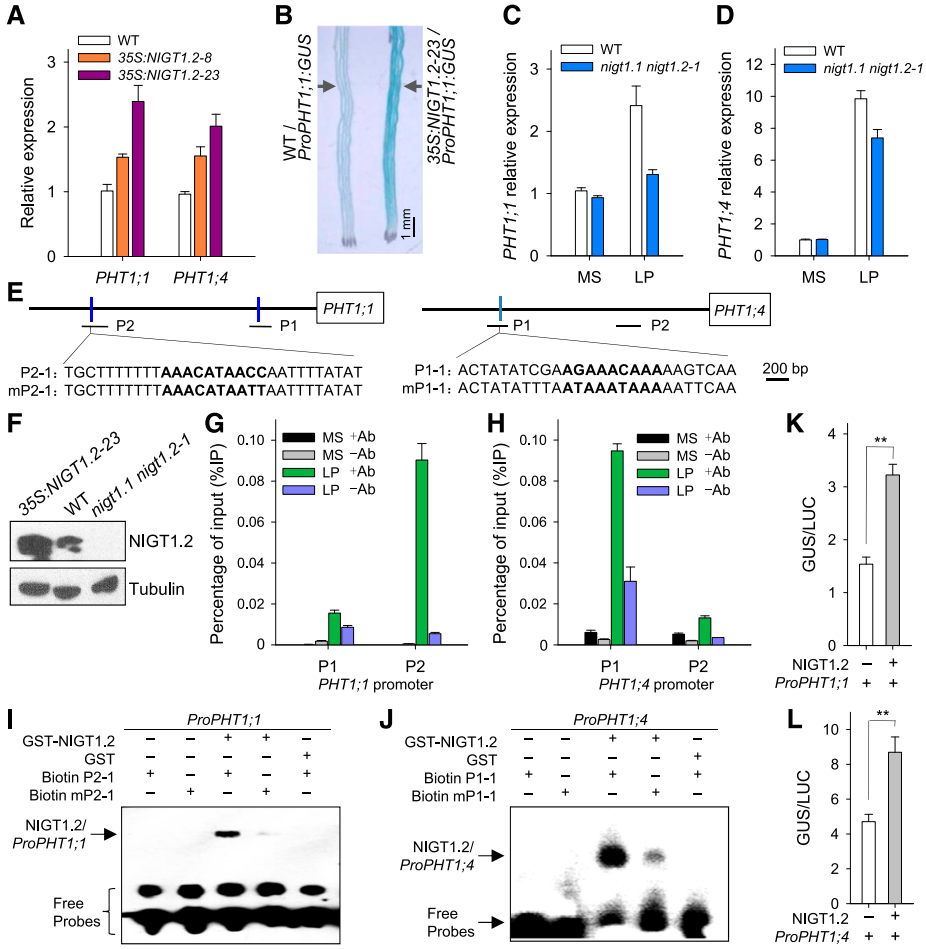

Figure 3. NIGT1.2 Directly Modulates Transcription of *PHT1;1* and *PHT1;4* in Response to Low-Pi Stress.

(A) RT-qPCR analysis of *PHT1;1* and *PHT1;4* in the roots of *NIGT1.2*-overexpressing lines and wild-type seedlings germinated and grown on MS medium for 10 d. Data are shown as mean ± SE (n = 3).

(B) GUS staining showing expression pattern of *PHT1;1* in roots of the *NIGT1.2*-overexpressing line and wild-type plants germinated and grown on MS medium for 7 d.

(C) and **(D)** RT-qPCR analysis of *PHT1;1* **(C)** and *PHT1;4* **(D)** in roots of 7-d-old *nigt1.1 nigt1.2* double mutant and wild-type seedlings grown on MS or LP medium for 5 d. Data are shown as mean ± SE (n = 3).

(E) Schematic representation of *PHT1;1* and *PHT1;4* promoter regions showing the relative positions of *cis*-regulatory elements (blue line). P1 and P2 indicate PCR fragments for ChIP-qPCR assay, and P2-1, mP2-1, P1-1, and mP1-1 are EMSA probes.

(F) Immunoblot analysis of NIGT1.2 protein using anti-NIGT1.2 antibody in *NIGT1.2*-overexpressing (*35S:NIGT1.2-23*), *nigt1.1 nigt1.2* double mutant, and wild-type plants grown on MS medium. Tubulin was used as the loading control.

(G) and **(H)** ChIP-qPCR assay of NIGT1.2 binding to promoters of *PHT1;1* **(G)** and *PHT1;4* **(H)** in vivo. Seven-day-old wild-type seedlings were grown on MS or LP medium for 5 d, and then roots were harvested for ChIP-qPCR assay using anti-NIGT1.2 antibody. Data are shown as mean ± SE (n = 3).

(I) and **(J)** EMSA of recombinant NIGT1.2 binding to promoters of *PHT1;1* **(I)** and *PHT1;4* **(J)** in vitro.

(K) and **(L)** Transient overexpression of *NIGT1.2* fused to *ProPHT1;1:GUS* **(K)** or *ProPHT1;4:GUS* **(L)** in *N. benthamiana* leaves. Data are shown as mean ± SE (n = 5). Asterisks in **(K)** and **(L)** indicate significant differences compared with wild-type plants (#) by Student's t test: *P < 0.05; **P < 0.01.

expression changes of *PHT1;1* and *PHT1;4* in the *nigt1.1 nigt1.2* double mutant during Pi starvation.

We next conducted an electrophoretic mobility shift assay (EMSA) to test whether NIGT1.2 bound to *cis*-elements within the *PHT1;1* and *PHT1;4* promoters. The NIGT1.2 recombinant protein produced an up-shift of the P2-1 probe on the P2 fragment of the *PHT1;1* promoter, and when the *cis*-element in the P2-1 probe was mutated from AAACATAACC to AAACATAATT, this up-shift was almost abolished (Figure 3I). The NIGT1.2 recombinant protein also bound to the P1-1 probe of the *PHT1;4* promoter (Figure 3J), and when the *cis*-element AGAAACAAA in the P1-1 of the *PHT1;4* promoter was mutated, the signal of the up-shifted NIGT1.2-mP1-1 complex was repressed (Figure 3J). These data demonstrate that NIGT1.2 can bind to promoters of *PHT1;1* and *PHT1;4* in vitro and in vivo.

To further test the function of NIGT1.2 in the regulation of *PHT1;1* and *PHT1;4* expression, we performed a transient

expression experiment in *Nicotiana benthamiana* leaves and found that NIGT1.2 up-regulated the activities of the *PHT1;1* and *PHT1;4* promoters (Figures 3K and 3L).

NIGT1.2 Negatively Modulates NO_3^- Uptake during Pi Starvation

We further measured N-related physiological parameters in the *NIGT1.2*-overexpressing lines and *nigt1.1 nigt1.2* double mutants under different Pi conditions. The N concentration was lower in *NIGT1.2*-overexpressing lines than in wild-type plants (Figure 4A). Moreover, the *35S:NIGT1.2-23* line, which had a higher transcript level of *NIGT1.2* than *35S:NIGT1.2-8* (Figure 2B), also showed lower N concentration (Figure 4A). The *nigt1.1 nigt1.2* double mutants displayed no obvious difference in N concentration as compared with wild-type plants when grown under Pi-sufficient conditions but showed a significantly increased N concentration under Pi-deficient conditions (Figure 4B). The N concentration of the *nigt1.2-1* or *nigt1.1* mutant was similar to that of wild-type plants under both Pi-sufficient and Pi-deficient conditions (Figure 4B).

Next, we measured the NO_3^- influx. The *NIGT1.2*-overexpressing lines displayed decreased NO_3^- influx compared with wild-type plants under Pi-sufficient conditions, and this decrease of NO_3^- influx was negatively dependent on *NIGT1.2* expression level (Figure 4C). The *nigt1.1 nigt1.2* double mutant showed no difference in NO_3^- influx from wild-type plants under Pi-sufficient conditions but significantly elevated NO_3^- influx under Pi-deficient conditions (Figure 4D). These data demonstrate that NIGT1.2 inhibits NO_3^- influx under low-Pi stress.

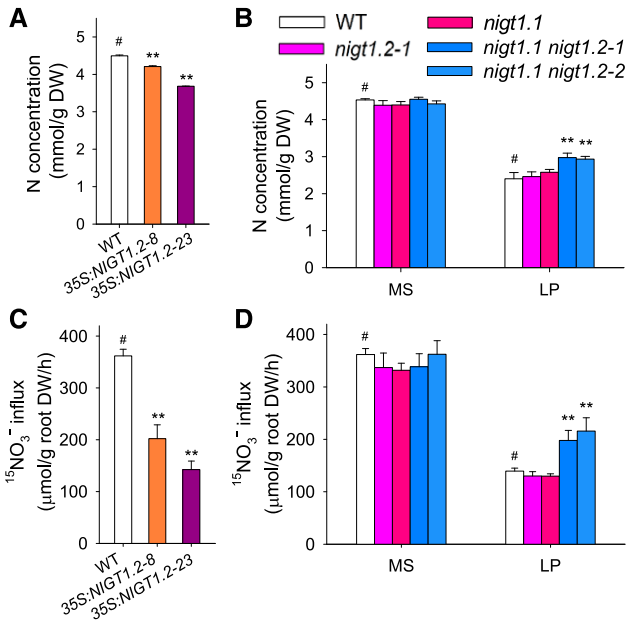

Figure 4. NIGT1.2 Reduces Arabidopsis Nitrate Influx under Low-Pi Stress.

(A) N concentration of 7-d-old *NIGT1.2*-overexpressing lines transferred to MS medium for another 7 d. Data are shown as mean ± SE ($n = 3$).

(B) N concentration of 7-d-old *nigt1.2-1*, *nigt1.1*, and *nigt1.1 nigt1.2* double mutants transferred to MS or LP medium for 7 d. Data are shown as mean ± SE ($n = 3$).

(C) $^{15}NO_3^-$ influx of 7-d-old *NIGT1.2*-overexpressing lines and wild-type plants transferred to MS medium for another 7 d. Data are shown as mean ± SE ($n = 4$).

(D) $^{15}NO_3^-$ influx of 7-d-old *nigt1.2-1*, *nigt1.1*, *nigt1.1 nigt1.2* double mutant and wild-type plants transferred to MS or LP medium for 7 d. Data are shown as mean ± SE ($n = 3$). Asterisks in **(A)**, **(B)**, **(C)**, and **(D)** indicate significant differences compared with wild-type plants (#) by Student's *t* test: *$P < 0.05$; **$P < 0.01$.

NIGT1.2 Directly Down-Regulates *NRT1.1* during Pi Starvation

We then measured *NRT1.1* expression in the *NIGT1.2*-overexpressing lines and the *nigt1.1 nigt1.2* double mutant. The transcription of *NRT1.1* was repressed in the *NIGT1.2*-overexpressing lines, and the degree of repression of *NRT1.1* was consistent with the *NIGT1.2* expression level (Figure 5A). We also crossed the *ProNRT1.1:GUS* transgenic line (Krouk et al., 2010) with wild-type plants and *35S:NIGT1.2-23*, and GUS staining results showed that NIGT1.2 repressed the activity of the *NRT1.1* promoter (Figure 5B). There was no difference in *NRT1.1* expression between *nigt1.1 nigt1.2* double mutant and wild-type plants under Pi-sufficient conditions (Figure 5C). During Pi starvation, however, the transcription of *NRT1.1* was repressed significantly in the wild-type plants but much less in the *nigt1.1 nigt1.2* double mutant (Figure 5C), suggesting that NIGT1.2 down-regulated transcription of *NRT1.1* under low-Pi stress. There was no difference in *NRT2.4* expression between *nigt1.1 nigt1.2* double mutant and wild-type plants under Pi-sufficient or Pi-deficient conditions (Figure 5C).

The transcription factor HRS1, the homolog of NIGT1.2, also binds to the core down-regulatory *cis*-element AGA (Medici et al., 2015). There were six down-regulatory *cis*-elements within the *NRT1.1* promoter (Figure 5D), and we hypothesized that NIGT1.2 down-regulated *NRT1.1* expression by binding to its promoter. We therefore conducted a ChIP assay on the roots of 7-d-old wild-type seedlings grown with or without low-Pi stress. In samples from seedlings grown under Pi-sufficient conditions, no NIGT1.2 enrichment was detected at the *NRT1.1* promoter fragments, P1 to P5, which contain one or two down-regulatory *cis*-regulatory elements; in contrast, in samples from Pi-deficient seedlings, the chromatin immunoprecipitated with anti-NIGT1.2 antibody was clearly enriched at the P5, P4, and P3 fragments of the *NRT1.1* promoter (Figure 5E), indicating that NIGT1.2 can bind to the *NRT1.1* promoter in vivo under low-Pi stress. We conducted the EMSA experiment to determine whether NIGT1.2 bound to down-regulatory *cis*-elements in these three fragments. The recombinant NIGT1.2 protein produced up-shifts for the P5-1, P4-1, P3-1, and P3-2 probes, each of which contains a *cis*-element, and when these *cis*-elements were mutated, the up-shift was virtually abolished (Figure 5F). These data demonstrate that NIGT1.2 binds to the *NRT1.1* promoter in vitro and in vivo.

To further confirm that NIGT1.2 directly modulated *NRT1.1* expression, we coexpressed *ProNRT1.1:GUS* with *35S:NIGT1.2* in *N. benthamiana* leaves. The NIGT1.2 significantly repressed

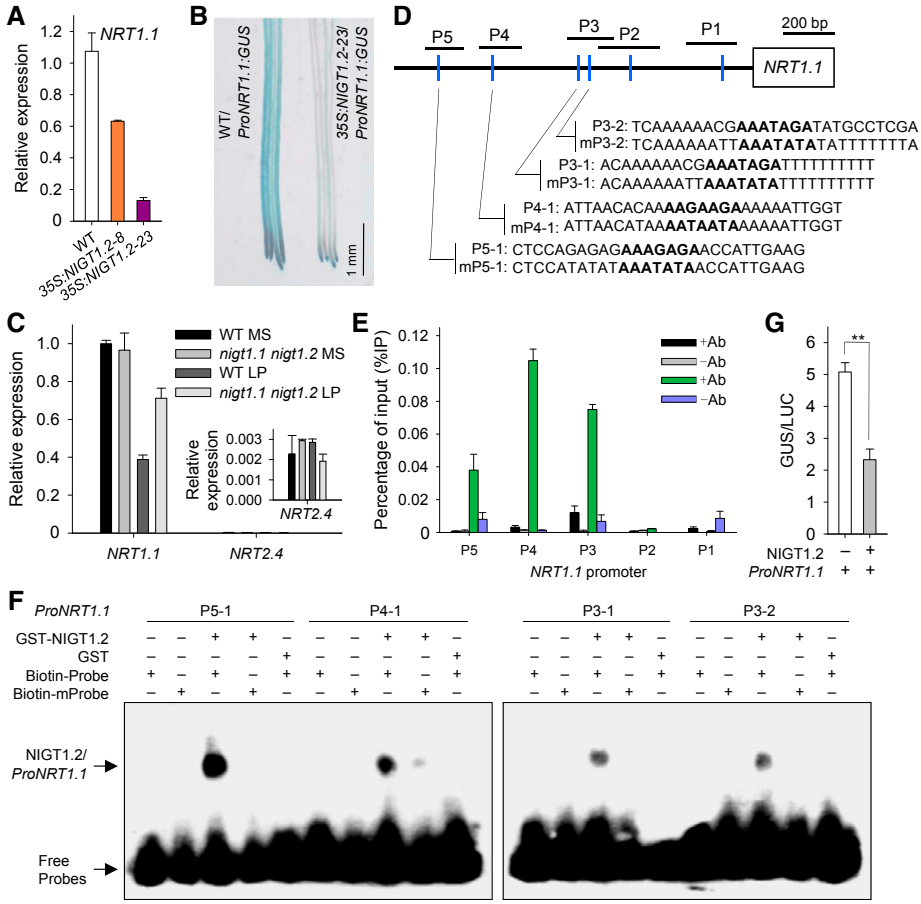

Figure 5. NIGT1.2 Directly Down-Regulates *NRT1.1* Expression in Response to Low-Pi Stress.

(A) RT-qPCR analysis of *NRT1.1* expression in roots of *NIGT1.2*-overexpressing lines and wild-type seedlings germinated and grown on MS medium for 10 d. Data are shown as mean ± SE (n = 3).

(B) GUS staining showing the expression pattern of *NRT1.1* in roots of *NIGT1.2*-overexpressing and wild-type plants germinated and grown on MS medium for 7 d.

(C) RT-qPCR analysis of *NRT1.1* and *NRT2.4* expression in roots of 7-d-old *nigt1.1 nigt1.2* double mutant and wild-type seedlings grown on MS or LP medium for 5 d. Data are shown as mean ± SE (n = 3).

(D) Schematic representation of *NRT1.1* promoter showing relative position of *cis*-element (blue line). P1 to P5 indicate PCR fragments for ChIP-qPCR, and P3-1 to P5-1 are EMSA probes.

(E) ChIP-qPCR assay of NIGT1.2 binding to *NRT1.1* promoter in vivo. Seven-day-old wild-type seedlings were transferred to MS or LP medium for 5 d, and then roots were harvested for ChIP-qPCR assay using anti-NIGT1.2 antibody. Data are shown as mean ± SE (n = 3).

(F) EMSA of recombinant NIGT1.2 binding to *NRT1.1* promoter in vitro.

(G) Transient overexpression of *NIGT1.2* fused to *ProNRT1.1:GUS* in *N. benthamiana* leaves. Data are shown as mean ± SE (n = 5). Asterisks indicate significant differences compared with wild-type plants (#) by Student's t test: **P < 0.01.

NRT1.1 promoter activity, compared with that in plants expressing *ProNRT1.1:GUS* alone (Figure 5G).

Epistatic Relationship between NIGT1.2 and *PHT1s* or *NRT1.1*

The *NIGT1.2*-overexpressing lines showed increased Pi uptake compared with wild-type plants (Figure 2), and NIGT1.2 positively regulated the transcription of *PHT1;1* and *PHT1;4* (Figure 3). The *pht1;1Δ4Δ* double mutant displayed a 75% reduction in Pi uptake capacity relative to wild-type plants, and the *pht1;1Δ4Δ* double mutant was in the Wassilewskija ecotype (Shin et al., 2004). To test the epistatic relationship between *NIGT1.2* and *PHT1;1/PHT1;4*, we generated the *pht1;1Δ4Δ 35S:NIGT1.2* line by overexpressing *NIGT1.2* in the *pht1;1Δ4Δ* double mutant (Figures 6A and 6B). The *NIGT1.2*-overexpressing line (*35S:NIGT1.2-8*) had an increased Pi uptake capacity and Pi concentration relative to wild-type plants of the ecotype Columbia (Col), whereas the *pht1;1Δ4Δ 35S:NIGT1.2* transgenic line displayed a reduced Pi uptake capacity and Pi concentration, similar to the *pht1;1Δ4Δ* double mutant (Figures 6C and 6D), indicating that*PHT1;1* and *PHT1;4* were genetically epistatic to *NIGT1.2*.

The molecular and biochemical assays showed that NIGT1.1/1.2 down-regulated *NRT1.1* expression under low-Pi stress, and the *nigt1.1 nigt1.2* double mutants displayed increased NO_3^- uptake rates and elevated expression of *NRT1.1* relative to wild-type plants during Pi starvation (Figures 4 and 5). Then we generated the *nigt1.1 nigt1.2 chl1-9* triple mutant (Figure 7A) by crossing the *nigt1.1 nigt1.2* double mutant with *chl1-9* mutant, which had a point mutation in *NRT1.1* resulting from Leu replacing Pro492 and was defective in both high- and low-affinity NO_3^- uptake (Ho et al., 2009). The NO_3^- uptake of wild-type plants was significantly reduced under low-Pi stress compared with that under Pi-sufficient conditions, whereas the *chl1-9* mutant showed a slightly reduced NO_3^- uptake under Pi-deficient conditions relative to that under Pi-sufficient conditions (Figure 7B), indicating that the NRT1.1 was a core NO_3^- transporter in response to Pi starvation. Disruption of *NRT1.1* in the *nigt1.1 nigt1.2* double mutant resulted in a decreased NO_3^- uptake, similar to the *chl1-9* mutant, under Pi-sufficient or Pi-deficient conditions (Figure 7B). These data suggest that NIGT1.1/1.2 modulate low-Pi-dependent NO_3^- uptake by down-regulating *NRT1.1* expression.

We also tested the phenotypes of the *nigt1.1 nigt1.2* double mutant under N and P combinatorial conditions. As shown in Figure 8A, the *nigt1.1 nigt1.2* double mutants were sensitive to low-Pi stress, with anthocyanin accumulation in leaves, compared with wild-type seedlings under −P+N conditions; when grown under +P−N conditions, the *nigt1.1 nigt1.2* double mutants showed N-deficient phenotypes with yellow leaves, similar to wild-type seedlings; and when grown under −P−N conditions, both the *nigt1.1 nigt1.2* double mutants and wild-type seedlings displayed N-deficient phenotypes, but not the Pi-deficient phenotypes. The biomasses of *nigt1.1 nigt1.2* double mutants were similar to those of wild-type plants under N and P combinatorial conditions (Figure 8B). Further, we tested the transcription of *NIGT1.1* and *NIGT1.2* under N and P combinatorial conditions. The RT-qPCR results showed that the expression of *NIGT1.1* and *NIGT1.2* was induced by low-Pi stress

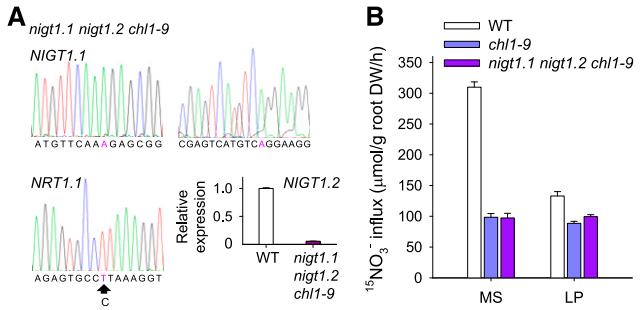

Figure 7. The *nigt1.1 nigt1.2 chl1-9* Triple Mutant Shows a Similar Nitrate Influx to the *chl1-9* Mutant.

(A) The mutation of the *nigt1.1 nigt1.2 chl1-9* triple mutant. The mutations in *NIGT1.1* and *NRT1.1* were evaluated by sequencing, and the mutant sites are indicated by pink letters. The expression of *NIGT1.2* in the *nigt1.1 nigt1.2 chl1-9* triple mutant was analyzed by RT-qPCR.
(B) $^{15}NO_3^-$ influx of 7-d-old seedlings transferred to MS or LP medium for 7 d. Data are shown as mean ± SE (n = 4).

(−P+N versus +P+N; −P−N versus +P−N), and these low-Pi inductions of *NIGT1.1* and *NIGT1.2* were independent of NO_3^- provision (Figure 8C). The transcription of *PHT1;1* and *PHT1;4* was increased under Pi-deficient conditions (−P+N versus +P+N; −P−N versus +P−N), and this increase was repressed in the *nigt1.1 nigt1.2* double mutant, typically under −P+N conditions (Figures 8D and 8E). *NRT1.1* expression was decreased in wild-type plants under −P+N conditions relative to that under +P+N conditions, and this decrease was weakened in the *nigt1.1 nigt1.2* double mutants (Figure 8F). Interestingly, the repression of *NRT1.1* by low-Pi stress was abolished in the *nigt1.1 nigt1.2* double mutants and wild-type plants under −P−N conditions relative to +P−N conditions (Figure 8F), indicating that the transcriptional regulation of *NRT1.1* was NO_3^- dependent.

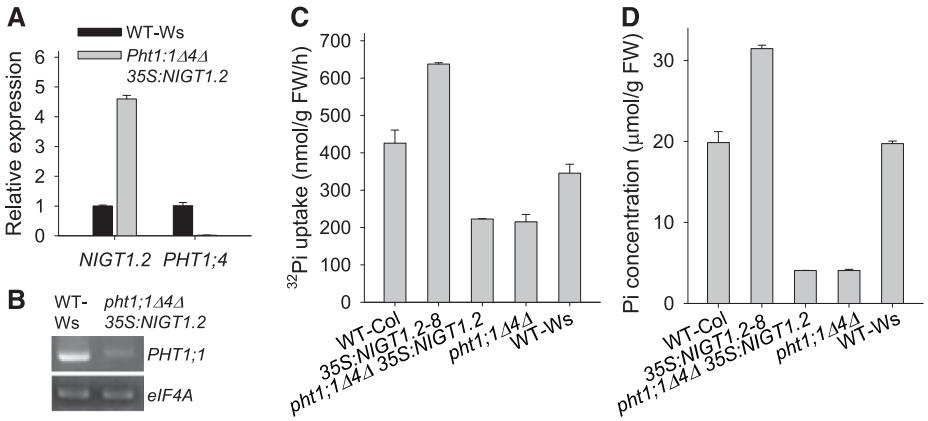

Figure 6. The *pht1;1Δ4Δ 35S:NIGT1.2* Transgenic Line Shows a Similar Pi Uptake Capacity to the *pht1;1Δ4Δ* Double Mutant.

(A) RT-qPCR analysis of *NIGT1.2* and *PHT1;4* in the *pht1;1Δ4Δ 35S:NIGT1.2* transgenic line. Data are shown as mean ± SE (n = 3).
(B) RT-PCR analysis of *PHT1;1* expression in the *pht1;1Δ4Δ 35S:NIGT1.2* transgenic line.
(C) ^{32}Pi uptake capacity of genotypes germinated and grown on MS medium for 10 d. Data are shown as mean ± SE (n = 4).
(D) Pi concentration of 7-d-old genotypes transferred to MS medium for 5 d. Data are shown as mean ± SE (n = 5).

Identification of NIGT1.2-Modulated Genes under Low-Pi Stress

To investigate the genome-wide transcriptional control exerted by NIGT1.2 during Pi starvation, we conducted an RNA sequencing experiment using roots from 7-d-old plants of wild-type Arabidopsis and a *NIGT1.2*-overexpressing line (OE23) grown on either standard MS medium or LP medium for 5 d. We identified differentially expressed genes (DEGs) relating to low-Pi stress, i.e., with expression that differed between the Pi-sufficient (MS) and Pi-deficient (LP) groups of wild-type plants, based on $P \leq 0.05$. We identified DEGs modulated by NIGT1.2, i.e., between the *NIGT1.2*-overexpressing line (OE23) and the wild-type plants, under Pi-sufficient conditions based on $P \leq 0.05$. In wild-type roots, we identified 3130 genes that were up-regulated and 2490 that were down-regulated during Pi starvation, and among these, 2179 (69.6%) up-regulated and 1920 (77.1%) down-regulated genes were modulated by constitutive overexpression of *NIGT1.2* (Figure 9A).

Among the DEGs modulated by NIGT1.2 under low-Pi stress, NIGT1.2 modulated genes related to diverse key cellular and metabolic functions (Figure 9B). Remarkably, 41 transporter genes were modulated by low-Pi stress via NIGT1.2, of which 24 were down-regulated, and 17 were up-regulated (Figures 9C and 9D). These 41 transporter genes encoded Pi transporters, NO_3^- transporters, amino acid transporters, calcium transporters, sugar transporters, and peptide transporters, among others (Figures 9C and 9D), suggesting that NIGT1.2 modulates transmembrane transport during Pi starvation. Further promoter sequence analysis showed that all 24 down-regulated transporter genes contained two or three down-regulatory *cis*-elements in their ~1500-bp promoters (Figure 9C), and all 17 up-regulated transporter genes had one or both up-regulatory *cis*-elements in their ~1500-bp promoters (Figure 9D), suggesting that NIGT1.2 might directly regulate the transcription of these transporter genes.

Interestingly, the genes modulated by the Pi starvation response and NIGT1.2 were enriched for functional classes of genes involving NO_3^- and amino acid transporters, nitrogen metabolism, carbohydrate (CHO)-metabolism and signaling (Figure 9B). Further analysis of the RT-qPCR analysis results showed that N-related genes encoding proteins from several major functional classes, including NO_3^- and amino acid transporters, amino acid and nitrogen metabolism, oxidative pentose-phosphate pathway, and CHO metabolism, were transcriptionally regulated by low-Pi stress and NIGT1.2 (Figure 9E), suggesting that NIGT1.2 plays important roles in regulating low-Pi-coupled NO_3^- responses. The

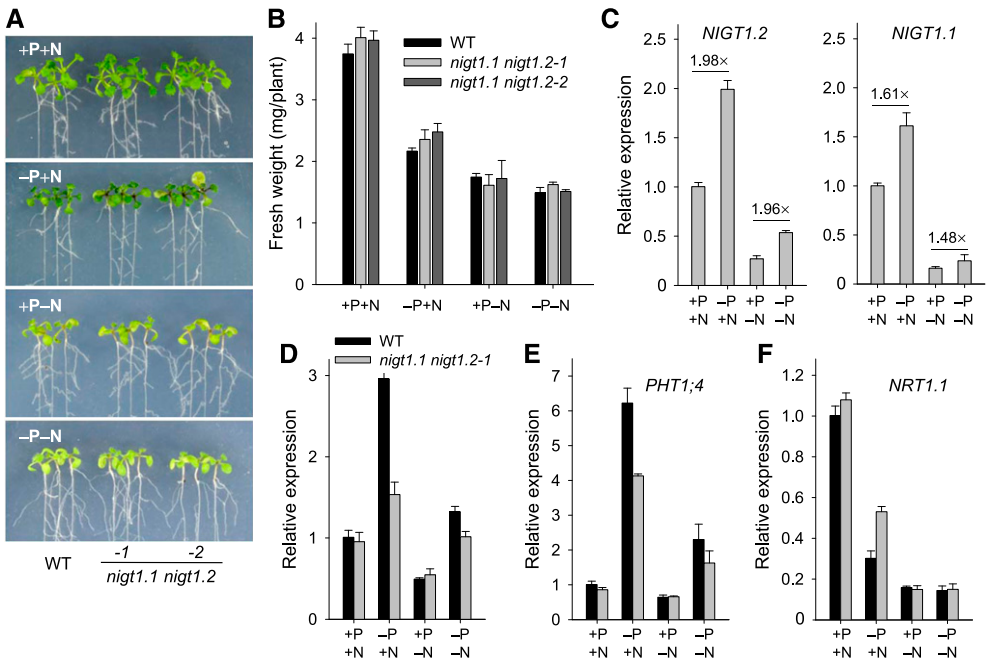

Figure 8. NIGT1.1 and NIGT1.2 Modulate the Transcription of *NRT1.1* and *PHT1s* in Response to a Combination of P and N Availability.

(A) Phenotypes of the *nigt1.1 nigt1.2* double mutants under N and P combinatorial conditions. Seven-day-old seedlings were transferred to MS medium (+P+N), without phosphate (−P+N), without nitrate (+P−N), or without phosphate and nitrate (−P−N) for 5 d, and then photographs were taken. The NH_4^+ in each medium was replaced with 0.5 mM ammonium succinate.

(B) Biomass analysis of the *nigt1.1 nigt1.2* double mutants and wild-type plants under N and P combinatorial conditions. Data are shown as mean ± SE (n = 3).

(C) RT-qPCR analysis of *NIGT1.2* and *NIGT1.1* in roots of 7-d-old wild-type Arabidopsis plants transferred to media with P/N combinations for 5 d. Data are shown as mean ± SE (n = 3).

(D) to **(F)** RT-qPCR analysis of *PHT1;1* **(D)**, *PHT1;4* **(E)**, and *NRT1.1* **(F)** in roots of 7-d-old *nigt1.1 nigt1.2* double mutant and wild-type Arabidopsis plants transferred to media with various P/N combinations for 5 d. Data are shown as mean ± SE (n = 3).

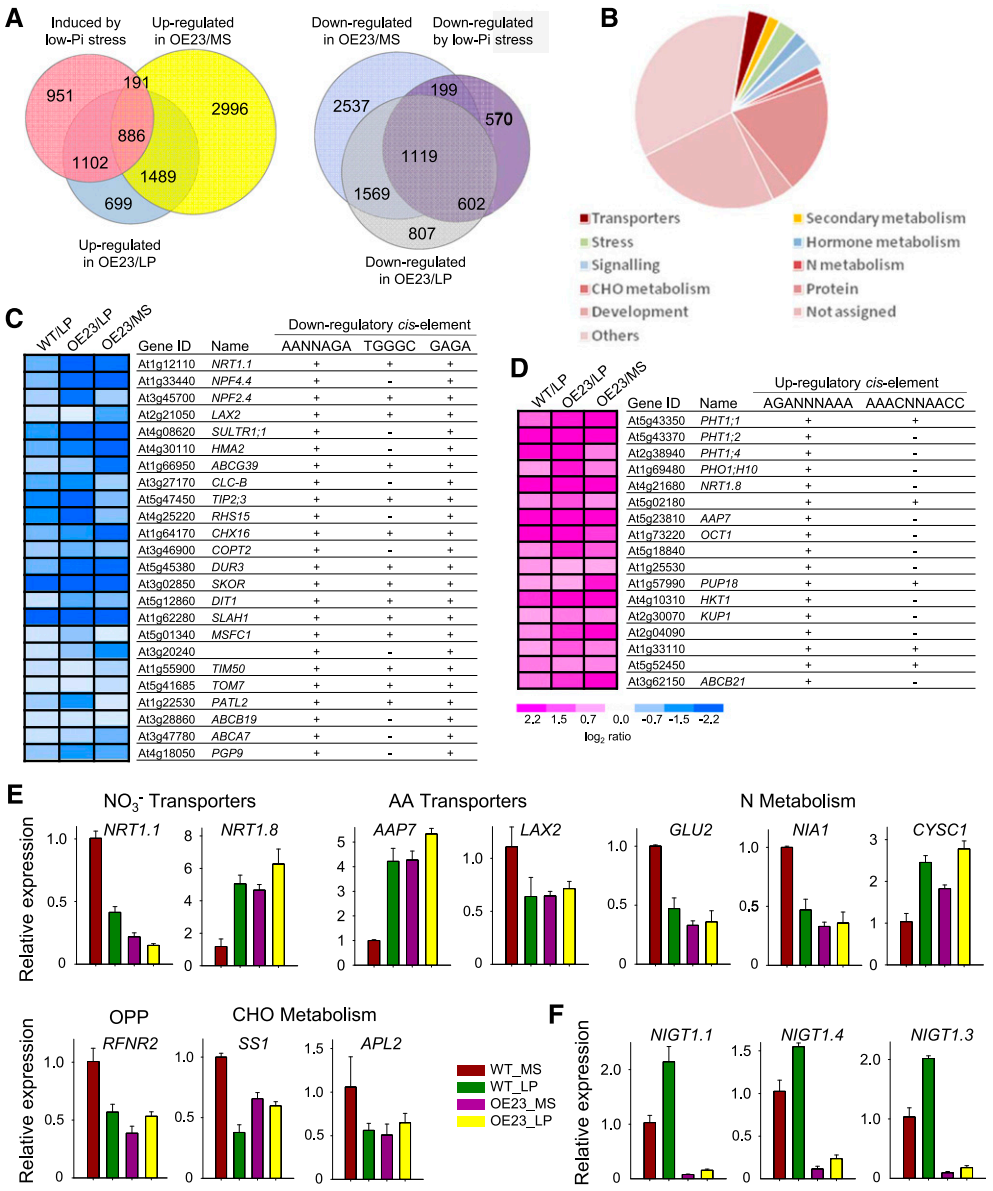

Figure 9. Identification of Target Genes of NIGT1.2.

(A) Venn diagram showing overlaps between genes induced by low-Pi stress and genes modulated by NIGT1.2. Seven-day-old *NIGT1.2*-overexpressing seedlings (OE23) and wild-type seedlings were transferred to MS or LP medium for 5 d, and then the roots were harvested for RNA-seq analysis.
(B) MapMan functional categories for low-Pi- and NIGT1.2-regulated genes.
(C) and **(D)** Hierarchical clustering analysis of transporter genes that were regulated by low-Pi stress and NIGT1.2 simultaneously.
(E) RT-qPCR analysis of some N-related genes encoding various categories of proteins, arbitrarily selected from among the NIGT1.2-regulated genes, in the roots of 7-d-old OE23 and wild-type seedlings grown on MS or LP medium for 5 d. Data are shown as mean ± SE ($n = 3$).
(F) RT-qPCR analysis of the three *NIGT1.2* homologs *NIGT1.1*, *NIGT1.4*, and *NIGT1.3* in the roots of 7-d-old OE23 and wild-type seedlings transferred to MS or LP medium for 5 d. Data are shown as mean ± SE ($n = 3$).

transcription of homologs of *NIGT1.2*, *NIGT1.1*, *HRS1/NIGT1.4*, and *NIGT1.3* was induced by low-Pi stress (Figure 9F), similar to what has previously been reported by Kiba et al. (2018) and Maeda et al. (2018). Moreover, the expression of all three of these genes was clearly repressed in the *NIGT1.2*-overexpressing line (Figure 9F).

Pi Deficiency Represses NO_3^- Uptake in Maize

Maize (*Zea mays*) is an important crop predominantly cultivated in soils where NO_3^- is often the primary source of nitrogen available for growth (Wen et al., 2017). When 9-d-old plants of the maize hybrid line B73 were transferred to hydroponic medium with (+P)

or without (–P) Pi for 11 d, their growth was repressed by Pi deficiency (Figures 10A and 10B). The P concentration was much lower in maize grown under Pi-deficient as compared with Pi-sufficient conditions (Figure 10C). Interestingly, the N concentration was also significantly lower in maize grown under Pi starvation as compared with Pi-sufficient conditions (Figure 10D), similar to what we observed in Arabidopsis (Figure 1). NO_3^- influx in maize was also reduced under Pi-deficient conditions (Figure 10E), suggesting that Pi deficiency decreased NO_3^- uptake in maize.

There are 79 members of the nitrate transporter/peptide transporter family (NRT1/PTR family, or NPF family) in maize B73, and eight members belong to NPF6 subfamily, named ZmNPF6.1 to ZmNPF6.8 (Figure 10F; Léran et al., 2014). Two *NPF6* genes, *ZmNPF6.4* and *ZmNPF6.6*, showed relatively high expression in roots (Figure 10G; maizeGDB, www.maizegdb.org). Their protein products ZmNPF6.4 and ZmNPF6.6, both homologs of Arabidopsis NRT1.1 (Figure 10F; Supplemental Data Set 1), function as NO_3^- transport proteins (Wen et al., 2017). ZmNPF6.4 displays a low-affinity NO_3^- transport activity in oocytes, and ZmNPF6.6 transports NO_3^- across a broad NO_3^- concentration range, including high- and low-affinity ranges (Wen et al., 2017). Because NO_3^- uptake was reduced in maize during Pi starvation (Figure 10E; de Magalhaes et al., 1998), we tested the transcription of *ZmNPF6.4* and *ZmNPF6.6* under Pi-deficient conditions. The RT-qPCR results showed that the transcription of both *ZmNPF6.4* and *ZmNPF6.6* was repressed in maize during Pi starvation (Figures 10H and 10I). There were several down-regulatory *cis*-elements (AANNAGA) in the *ZmNPF6.4* and *ZmNPF6.6* promoters, suggesting that *ZmNPF6.4* and *ZmNPF6.6* were transcriptionally regulated by the maize homolog of transcription factor AtNIGT1.2.

The maize genome contains two homologs of *AtNIGT1.2*, which we named *ZmNIGT1.1* (Zm00001d023402) and *ZmNIGT1.2* (Zm00001d023411), and these two *ZmNIGT1* genes had 94.8% identity of coding sequence. The transcription of both genes was induced in maize roots during Pi starvation (Figure 10J). Further, ZmNIGT1.2 repressed the activity of the *ZmNPF6.6* promoter during transient expression in *N. benthamiana* (Figure 10K), suggesting that ZmNIGT1.2 down-regulated the transcription of *ZmNPF6.6*.

Discussion

In this study, we uncovered a NIGT1.2- and NIGT1.1-dependent regulatory pathway mediating the antagonistic cross talk between Pi and NO_3^- uptake in plants under low-Pi stress. These findings allow us to propose a working model for low-Pi-stress-related Pi and NO_3^- uptake modulation by these two transcription factors (Figure 11).

As a transcription factor, NIGT1.2 was localized in the nucleus, and its transcription was induced during Pi starvation (Supplemental Figure 1; Kiba et al., 2018), which was modulated by the core transcription factor PHR1 (Bustos et al., 2010; Maeda et al., 2018). Overexpression of *NIGT1.2* enhanced the Pi uptake capacity (Figure 2) but reduced NO_3^- uptake capacity (Figure 4) in Arabidopsis, and the *nigt1.1 nigt1.2* double mutants displayed decreased Pi but increased NO_3^- uptake capacity under low-Pi stress (Figures 2 and 4), demonstrating that NIGT1.2 modulated the uptake of Pi and NO_3^- during Pi starvation. In Arabidopsis, PHT1;1 and PHT1;4 are the main Pi transporters functioning in Pi uptake under both Pi-sufficient and Pi-deficient conditions (Shin et al., 2004), and NRT1.1 functions as an important dual-affinity NO_3^- transporter involved in multiple phases of NO_3^- uptake (Liu et al., 1999; Wang et al., 2012). Pi starvation induced *PHT1;1* and *PHT1;4* transcription (Figures 3C and 3D; Shin et al., 2004) but repressed *NRT1.1* expression (Figure 1F). EMSA and ChIP analysis demonstrated that during low-Pi stress, NIGT1.2 directly bound to the promoters of *PHT1;1*, *PHT1;4*, and *NRT1.1*, and RT-qPCR results showed that under low-Pi conditions, NIGT1.2 up-regulated the transcription of *PHT1;1* and *PHT1;4* but down-regulated that of *NRT1.1* (Figures 3 and 5), indicating that NIGT1.2 modulated Pi-dependent antagonistic absorption of Pi and NO_3^- by directly regulating the transcription of *PHT1;1*, *PHT1;4*, and *NRT1.1*.

Kiba et al. (2018) found that besides the N starvation-responsive genes, NIGT1s also modulated the expression of SPX DOMAIN genes (*SPXs*) and *PHOSPHATE2* (*PHO2*), which encode main regulators in response to Pi starvation (Liu et al., 2012; Puga et al., 2014). The transcription of *SPX1*, *SPX4*, and *PHO2* was elevated in the NIGT1.2ox line under N sufficiency and decreased in the NIGT1.2ox under N starvation conditions, compared with wild-type Arabidopsis (Kiba et al., 2018). We then measured the transcription of *SPXs* and *PHO2* in the *nigt1.1 nigt1.2* double mutant under Pi starvation conditions. The expression of *SPX1* and SPX4 in the *nigt1.1 nigt1.2* double mutant was similar to that in wild-type plants under either Pi-sufficient or Pi-deficient conditions (Supplemental Figures 5A and 5B). The *PHO2* expression was slightly reduced in the *nigt1.1 nigt1.2* double mutant relative to that in wild-type plants under Pi-sufficient conditions and similar between the *nigt1.1 nigt1.2* double mutant and wild-type under Pi-deficient conditions (Supplemental Figure 5C). These data suggest that NIGT1.1/1.2 modulate the transcription of *SPXs* and *PHO2* in response to N starvation, and to a lesser extent in response to P starvation.

In addition to our work in Arabidopsis, we also tested NO_3^- uptake in maize under low-Pi stress. N concentration and NO_3^- influx were reduced in maize under Pi-deficient conditions (Figure 10), as previously reported by de Magalhaes et al. (1998). ZmNPF6.4 and ZmNPF6.6, two homologs of ArabidopsisNRT1.1, showed NO_3^- transport activities under nitrate-sufficient conditions (Wen et al., 2017), and their transcription was repressed during Pi starvation (Figures 10H and 10I), indicating that Pi deficiency repressed maize NO_3^- absorption by repressing the transcription of NO_3^- transporter genes. ZmNIGT1.1 and ZmNIGT1.2, two homologs of AtNIGT1.2, were transcriptionally induced during Pi starvation and in turn repressed the promoter activity of *ZmNPF2* in *N. benthamiana* assays (Figures 10J and 10K). These data indicate that the Pi-dependent NIGT1.1/1.2-NRT/NPF regulatory pathway exists in crop plants as well as in the model plant Arabidopsis.

Previous reports showed that NIGT1 proteins function as transcriptional repressors of the *NRT2.1* and *NRT2.4* promoters (Kiba et al., 2018; Maeda et al., 2018). In this work, NIGT1.2 acted as both an activator and repressor (Figures 3 and 5), similar to proteins such as HRS1/NIGT1.4, NLP7, WRKY6, and WRKY42

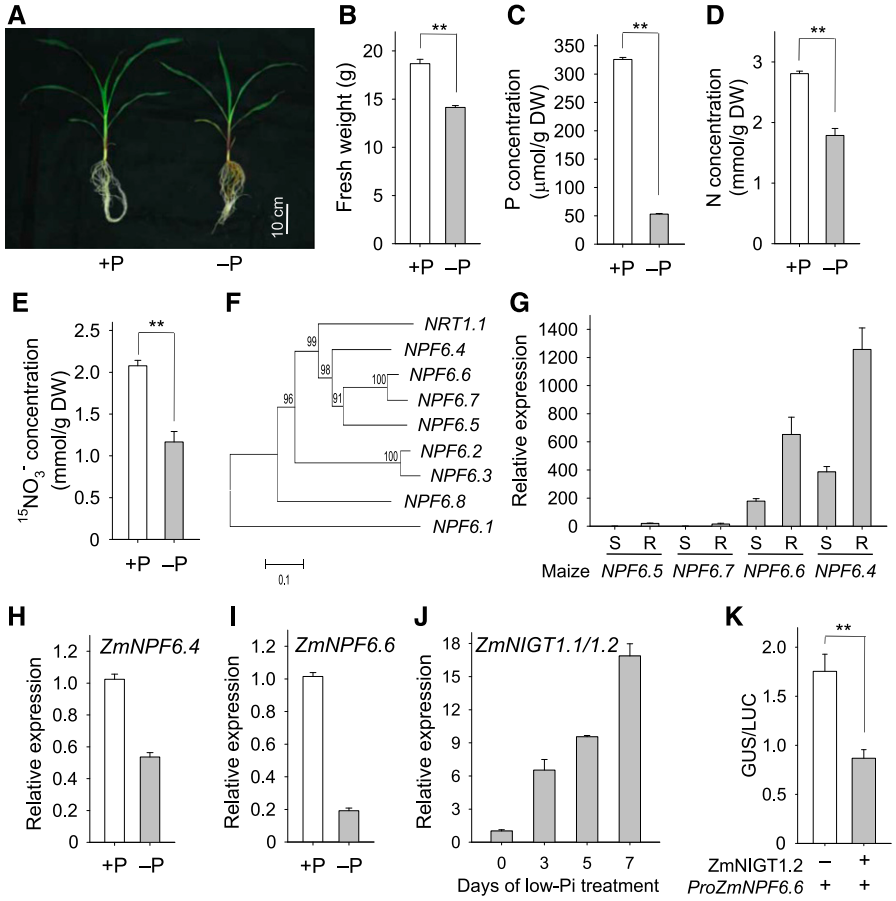

Figure 10. Pi Deficiency Represses Maize Nitrate Influx.

(A) Phenotypic comparison of maize inbred B73 grown under Pi-sufficient and Pi-deficient conditions. Nine-day-old maize B73 plants were transferred to one-eighth modified Hoagland nutrient solution with (+P) or without Pi (−P) for 11 d.

(B) to **(D)** Biomass **(B)**, phosphorus concentration **(C)**, and nitrogen concentration **(D)** of 9-d-old maize B73 transferred to +P or −P solution for 11 d. Data are shown as mean ± SE ($n = 5$).

(E) Nitrate concentration of 9-d-old maize B73 transferred to +P or −P solution with 5 mM NO_3^- (5 atom% ^{15}N) nitrate for 11 d. Data are shown as mean ± SE ($n = 5$).

(F) Neighbor-joining tree analysis was conducted using MEGA6.

(G) RT-qPCR analysis of maize nitrate transporter gene *NPFs* in shoots **(S)** and roots **(R)**. Data are shown as mean ± SE ($n = 4$).

(H) and **(I)** RT-qPCR analysis of *ZmNPF6.4* **(H)** and *ZmNPF6.6* **(I)** in roots of 9-d-old maize B73 seedlings transferred to +P or −P solution for 5 d.

(J) RT-qPCR analysis of *ZmNIGT1.1/1.2* in roots of maize B73 during Pi starvation. Data are shown as mean ± SE ($n = 4$).

(K) Transient expression of *ZmNIGT1.2* fused to *ProNPF2:GUS* in *N. benthamiana* leaves. Data are shown as mean ± SE ($n = 5$). Asterisks in **(B)** to **(E)** and **(K)** indicate significant differences compared with wild-type plants (#) by Student's *t* test: **P < 0.01.

(Robatzek and Somssich, 2002; Chen et al., 2009b; Castrillo et al., 2013; Marchive et al., 2013; Medici et al., 2015; Su et al., 2015). According to previous reports, the function of a transcription factor as an activator or a repressor presumably was determined by the structure of the promoter, the binding sites, the flanking sequences, and its interplay regulator (Blauwkamp et al., 2008; Medici et al., 2015). NIGT1.2 can bind to two types of *cis*-motifs, AGANNNAAA and AAACNNAACC, in the promoters of *PHT1;1* and *PHT1;4*, which are up-regulated by NIGT1.2; AANNAGA, TGGGA, and GAGA in the promoter of *NRT1.1*, which is down-regulated by NIGT1.2 (Figures 3 and 5), similar to HRS1/NIGT1.4 (Medici et al. 2015). Furthermore, the binding of NIGT1.2 to the *PHT1;4* promoter was sequence specific and displayed different requirements for the sequences flanking this motif (Figures 3E and 3J). These data suggest that the NIGT1 proteins, such as NIGT1.2 and NIGT1.4, function as transcriptional repressors or activators, at least partially through binding to different *cis*-motifs.

Besides modulating Pi and NO_3^- uptake, NIGT1.2 also modulated the transcription of N-response genes, including genes related to amino acid metabolism, amino acid transport, and N metabolism, and those encoding oxidative pentose-phosphate pathway, CHO, and N-response transcription factors (Figure 9). Notably, these genes were transcriptionally modulated during Pi starvation, and most of their promoters contained *cis*-regulatory elements for NIGT1.2 binding (Figure 9), suggesting that NIGT1.2 modulated N-related cellular metabolism under low-Pi stress.

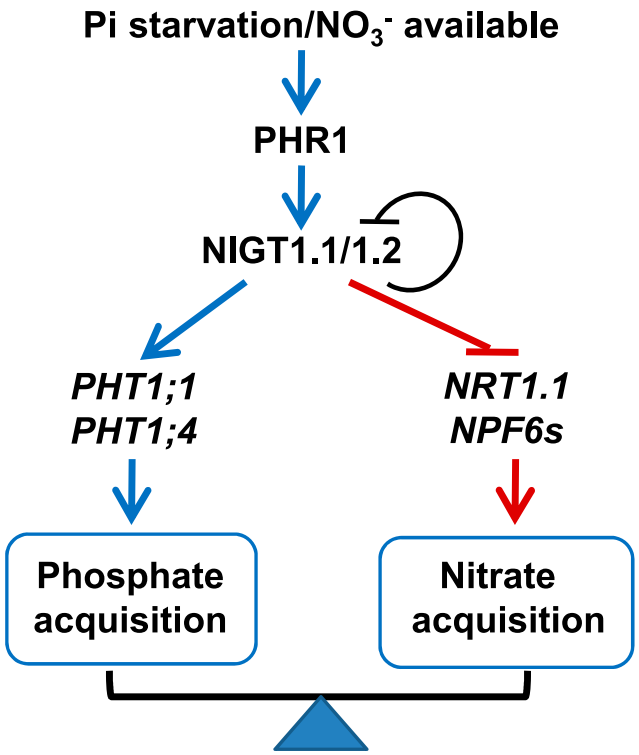

Figure 11. A Model of NIGT1.1/1.2-PHT1s/NRTs-Regulatory Pathway in Plant Regulating Phosphate and Nitrate Uptake.

Under Pi-deficient and NO_3^--sufficient conditions, NIGT1.1 and NIGT1.2 have a dual role both as direct activators of Pi transporters and as direct repressors of NO_3^- transporters to balance N and P uptake.

NIGT1.2 transcription was also up-regulated by NO_3^- (Supplemental Figure 1B; Medici et al., 2015; Kiba et al., 2018; Maeda et al., 2018), and *NIGT1.2* overexpression increased Pi uptake but repressed NO_3^- uptake (Figures 2 and 4), indicating that NIGT1.2 participates in Pi and NO_3^- influx under N-sufficient conditions. NIGT1.2 directly activated the transcription of both *PHT1;1* and *PHT1;4* (Figure 3), which encode two main Pi transporters involved in root Pi transport under both Pi-sufficient and Pi-deficient conditions (Shin et al., 2004). NIGT1.2 directly down-regulated the transcription of *NRT1.1* (Figure 5), which encodes an important dual-affinity NO_3^- transporter that functions under both N-sufficient and N-deficient conditions (Tsay et al., 1993; Liu et al., 1999; Liu and Tsay, 2003; Wang et al., 2012, 2018) and transcriptionally down-regulated *NRT2.1* (Maeda et al., 2018) and *NRT2.4* (Kiba et al., 2018), which encode two typical high-affinity NO_3^- transporters that function mainly under N-deficient conditions (Filleur et al., 2001; Orsel et al., 2004; Li et al., 2007; Kotur et al., 2012; Wang et al., 2012, 2018). NIGT1.2 (also named HHO2) also modulated the number and length of lateral roots irrespective of the Pi availability (Nagarajan et al., 2016), which may influence the Pi and NO_3^- uptake. Then, NIGT1.1 and NIGT1.2 may play important roles in increasing Pi and NO_3^- uptake under conditions of fluctuating NO_3^- and Pi availability.

Recently, Medici et al. (2019) and Hu et al. (2019) demonstrated that nitrogen, mainly NO_3^-, triggers the phosphate starvation response (PSR). Nitrate triggers phosphate starvation-induced gene expression through the SPX4-PHR2 module (Hu et al., 2019), PSR strongly depends on N provision, and N provision modulates PHR1accumulation and turnover (Medici et al., 2019). *NIGT1.2* expression was induced by NO_3^- (Supplemental Figure 1; Kiba et al., 2018) and was modulated by the transcription factor PHR1 (Bustos et al., 2010; Maeda et al., 2018), suggesting that the transcription factor NIGT1.2 participates in the nitrate-dependent PSR response. Ueda and Yanagisawa (2019) also indicated that the NIGT1s, including the NIGT1.1 and NIGT1.2, participate in integrating NO_3^- and Pi signals from various environment factors and triggering appropriate responses. Our work provides insight into NIGT1.1- and NIGT1.2-modulated low-Pi-stress-related phosphate and nitrate uptake.

METHODS

Plant Materials

The Col-0 ecotype was used as wild-type Arabidopsis (*Arabidopsis thaliana*) in this study. The T-DNA insertion lines GK_122B12, Salk_137632, and Salk_070096 (referred to as the *nigt1.2-1*, *nigt1.2-2*, and *nigt1.2-3* mutants) were ordered from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/abrc). The *ProPHT1;1:GUS* (Wang et al., 2014) and *ProNRT1.1:GUS* (Krouk et al., 2010) line were described previously. To construct *35S:NIGT1.2*, the coding sequence of *NIGT1.2* was amplified by PCR using gene-specific primers (Supplemental Table) and cloned into the *pCXSN* vector (Chen et al., 2009a). A pair of small guide RNA targets (M1, ATAATGATGATGTTCAAGAGCGG; M2, AGCTATCGA GTCATGTCGGAAGG) in *NIGT1.1* was cloned into the *pHEE2A-TRI* vector (Wang et al., 2015), and transformed into wild-type Arabidopsis and *nigt1.2-1* to generate the *nigt1.1* mutant and *nigt1.1 nigt1.2* double mutant, respectively. All constructs were transformed into plants via floral dip transformation (Clough and Bent, 1998).

Plant Growth Conditions

Arabidopsis seeds were stratified at 4°C for 72 h and then germinated and grown on MS medium at 22°C with a 16-h-fluorescent daily light period (light intensity 100μmol/m^2s). The LP medium was made by modifying MS medium to contain only 10 μM Pi, as well as agar instead of agarose (Promega). For Arabidopsis hydroponic culture, the seedlings were grown in one-fourth MS nutrient solution without supplement of Suc. For maize (*Zea mays*) hydroponic culture, 9-d-old maize seedlings without endosperm were transferred to one-eighth Hoagland nutrient solution with or without 250 μM KH_2PO_4, and grown at 28°C with a 14-h-fluorescent daily light period (light intensity 350 μmol/m^2s).

Physiological Measurements

The Pi concentration and Pi uptake measurements for Arabidopsis and maize seedlings were quantified as previously described by Su et al. (2015). The nitrogen and $^{15}NO_3^-$ concentration in Arabidopsis and maize were modified from previously described by Hu et al. (2015). For measurements of nitrogen and $^{15}NO_3^-$ concentration in Arabidopsis, the 7-d-old seedlings grown on MS were transferred to modified LP or MS medium containing 5 mM NO_3^- (5 atom% ^{15}N) and the NH_4NO_3 was replaced by ammonium succinate. For nitrogen and $^{15}NO_3^-$ concentration in maize, 9-d-old maize B73 plants were transferred to +P or −P solution with 5 mM NO_3^- (5 atom% ^{15}N) for 11 d. Whole seedlings were dried at 80°C for 3 d and analyzed using isotope ratio mass spectrometry (EA-DELTA^{plus} XP).

For NO_3^- influx assay, 7-d-old Arabidopsis seedlings were grown on LP or MS medium for another 7 d. The plants were washed with 0.1 mM $CaSO_4$ for 1 min, and then transferred to uptake solution for 5 min. The uptake

solution was MS solution containing 20 mM NO_3^- (99 atom% ^{15}N) and 0.5 mM ammonium succinate, without NH_4NO_3 and agar. Roots were washed with 0.1 mM $CaSO_4$ for 1 min and dried at 80°C for 3 d and then analyzed using isotope ratio mass spectrometry (EA-DELTA^{plus} XP). Influx of $^{15}NO_3^-$ was calculated from the total N and ^{15}N concentration in roots.

GUS Staining and Subcellular Localization

A GUS staining assay was performed as previously described by Chen et al. (2009b). For the subcellular localization experiment, *NIGT1.2* fused to GFP was cloned into a modified *pCAMBIA1300:GFP* vector, resulting in a *NIGT1.2-GFP* construct. The *NIGT1.2-GFP* construct and *GFP* alone were each transformed into leaves of *Nicotiana benthamiana* through transient expression assays as previously described by Chen et al. (2009b). GFP fluorescence in the transformed leaves was imaged using a confocal laser scanning microscope (LSM510, Carl Zeiss).

Transient Expression Assay in *N. benthamiana*

The transient expression assay was performed as previously described by Chen et al. (2009b). The *ProPHT1;1:GUS* construct was previously described by Wang et al. (2014). To construct *ProNRT1.1* and *ProZmNPF2,* the ~1.5 kb of the promoter regions of *NRT1.1* and *ZmNPF2* were cloned into the *pCAMBIA1381* vector. To construct *35S:ZmNIGT1.2*, the coding sequence of *ZmNIGT1.2* was cloned into the *pCXSN* vector. The primer sequences used are listed in the Supplemental Table. *Super:LUC* was added as an internal control in each infiltration sample. The GUS and LUC activities were measured in each infiltrated sample, and the GUS/LUC ratio was used to quantify the promoter activity.

RT-qPCR Analysis

RT-qPCR analysis was conducted as previously described by Huang et al. (2016). Relative quantitative results were calculated by normalization to *Actin2/8* in Arabidopsis and to *Ubiquitin* (*ZmUBQ*; GenBank accession number: BT018032) in maize. Each experiment was performed in biological triplicate. The primers used are listed in the Supplemental Table.

RNA Sequencing Analysis

Arabidopsis plants were grown as described above, with pooled roots from 120 seedlings used for each of three independently grown and harvested biological replicates. For each biological replicate, 3 μg of rRNA-depleted RNA was used for cDNA conversion and an Illumina sequencing library was generated using the RNA library prep kit for Illumina (NEB). After sequencing, Illumina adapter sequences were aligned to the Arabidopsis genome TAIR10 using TopHat2 (Kim et al., 2013), and differential expression analysis was performed using DESeq2 (Love et al., 2014). The raw data were submitted to the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/sra/PRJNA625449; no. PRJNA625449). DEGs were determined for wild type_MS versus wild type_LP and wild type_MS versus OE23_MS with a $P \leq 0.05$ cut-off. The function of enriched genes was analyzed using the Classification SuperViewer Tool on the BAR website (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi) with the MapMan classification source option. A heatmap was generated with Gene Cluster 3.0 (de Hoon et al., 2004) and visualized with Java Treeview (Saldanha, 2004).

Protein Expression and Antibody Generation

The coding sequence of *NIGT1.2* was cloned into the *pGEX-4T-1* vector to produce the *GST-NIGT1.2* vector, and then *GST-NIGT1.2* was introduced into and expressed in *Escherichia coli* strain BL21. The *E. coli* cells were induced with 0.2 mM isopropyl-D-1-thiogalactopyranoside overnight at 18°C and collected by centrifugation. The polyclonal antibody against NIGT1.2 was generated by inoculating mice.

ChIP-qPCR and EMSA

For ChIP-qPCR, 7-d-old wild-type Arabidopsis seedlings were transferred to MS or LP medium for 5 d, and then the roots were harvested for the ChIP experiment. The ChIP experiment was performed using anti-NIGT1.2 antibody as previously described by Chen et al. (2009b) and Huang et al. (2016). EMSA was conducted as previously described by Huang et al. (2016). The primers used are listed in the Supplemental Table.

Phylogenetic Analysis

The Arabidopsis NRT1.1 and maize NPF sequences were retrieved from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) database. For the phylogenetic analysis, the amino acid sequences were aligned in ClustalX (version 2.0.11) with default parameters. Bootstrap values were obtained based on 900 replicates. Evolutionary analysis was conducted in MEGA6 software.

Statistical Analysis

Data are shown as mean ± SE of one representative experiment. Student's *t* test was used to compare significance between treatment or genotypes. P value was shown as $P < 0.05$ or $P < 0.01$ to indicate significant difference.

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under the following accession numbers: *NIGT1.1* (AT1G25550), *NIGT1.2* (AT1G68670), *PHT1;1* (AT5G43350), *PHT1;4* (AT2G38940), *NRT1.1* (AT1G12110), *NRT1.2* (AT1G69850), *NRT2.1* (AT1G08090), *NRT2.4* (AT5G60770), *ZmNIGT1.1* (Zm00001d023411), *ZmNIGT1.2* (Zm00001d023402), *ZmNPF6.4* (Zm00001d024587), and *ZmNPF6.6* (Zm00001d029932).

Supplemental Data

Supplemental Figure 1. *NIGT1.2* expression pattern.

Supplemental Figure 2. Identification of *nigt1.2* mutants and Pi concentration measurement.

Supplemental Figure 3. Analysis of *NIGT1.1* expression using RT-qPCR.

Supplemental Figure 4. Generation of the *nigt1.1* and *nigt1.1 nigt1.2* double mutant using CRISPR/Cas9 technology.

Supplemental Figure 5. Analysis of *SPXs* and *PHO2* expression using RT-qPCR.

Supplemental Table. Primer sequences used in this study.

Supplemental Data Set 1. Alignments used to generate the phylogeny presented in Figure 10F.

Supplemental Data Set 2. Student's *t* test tables.

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AUTHOR CONTRIBUTIONS

Y.-F.C. designed the project; X.W., H.-F.W., Y.C., and M.-M.S. performed the experiments; Y.-F.C. and X.W. analyzed the data and wrote the article.

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