

# Interacting TCP and NLP transcription factors control plant responses to nitrate availability

Peizhu Guan<sup>a</sup>, Juan-José Ripoll<sup>a</sup>, Renhou Wang<sup>a</sup>, Lam Vuong<sup>a</sup>, Lindsay J. Bailey-Steinitz<sup>a</sup>, Dening Ye<sup>b,1</sup>, and Nigel M. Crawford<sup>a,2</sup>

<sup>a</sup>Division of Biological Sciences, Section of Cell and Developmental Biology, University of California, San Diego, La Jolla, CA 92093; and <sup>b</sup>Department of Chemistry and Biochemistry, Ohio University, Athens, OH 45701

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Plants have evolved adaptive strategies that involve transcriptional networks to cope with and survive environmental challenges. Key transcriptional regulators that mediate responses to environmental fluctuations in nitrate have been identified; however, little is known about how these regulators interact to orchestrate nitrogen (N) responses and cell-cycle regulation. Here we report that teosinte branched1/cycloidea/proliferating cell factor1-20 (TCP20) and NIN-like protein (NLP) transcription factors NLP6 and NLP7, which act as activators of nitrate assimilatory genes, bind to adjacent sites in the upstream promoter region of the nitrate reductase gene, *NIA1*, and physically interact under continuous nitrate and N-starvation conditions. Regions of these proteins necessary for these interactions were found to include the type I/II Phox and Bem1p (PB1) domains of NLP6&7, a protein-interaction module conserved in animals for nutrient signaling, and the histidine- and glutamine-rich domain of TCP20, which is conserved across plant species. Under N starvation, TCP20-NLP6&7 heterodimers accumulate in the nucleus, and this coincides with TCP20 and NLP6&7-dependent up-regulation of nitrate assimilation and signaling genes and down-regulation of the G<sub>2</sub>/M cell-cycle marker gene, *CYCB1;1*. TCP20 and NLP6&7 also support root meristem growth under N starvation. These findings provide insights into how plants coordinate responses to nitrate availability, linking nitrate assimilation and signaling with cell-cycle progression.

TCP | NIN-like protein | nitrate signaling | cell cycle | root growth

Nitrate is the main form of inorganic nitrogen (N) in aerobic soils and is usually the most growth-limiting plant nutrient. Nitrate is also a potent signal that regulates plant metabolism, growth, and development. As sessile organisms, plants have evolved an elaborate regulatory network in response to nitrate, which can fluctuate spatiotemporally in soil solution by up to four orders of magnitude (1–3). In *Arabidopsis*, nitrate induces the primary nitrate response (PNR) in roots and shoots, where rapid, broad-ranged modulation of gene expression affects over 1,000 genes (4, 5). Using a nitrate reductase-null mutant, our previous work further showed that 595 genes responded to nitrate alone, independent of nitrate reduction. Those genes are most overrepresented in the categories of energy and metabolism, including glycolysis and gluconeogenesis, amino acid metabolism, nitrogen and sulfur utilization, and transport facilitation (6). Furthermore, the PNR is accompanied by adaptive processes in nitrate transport activity and remobilization and modulations of root growth (2).

Underlying N-regulated, adaptive responses in metabolism and development are transcription factors (TFs), which play crucial roles in regulating nitrate-responsive genes, particularly the sentinel genes such as *NPF6.3* (*CHL1/NRT1.1*), *NRT2.1*, *NIA1*, *NIA2*, or *NiR* (2, 7). Many TFs, including *ANRI*, *NLP6/7*, *LBD37/38/39*, *SPL9*, *NAC4*, *bZIP1*, *TGA1/4*, *TCP20*, and *NRG2*, have been thus far identified in mediating nitrate responses (8–17). However, the transcriptional mechanisms that coordinate the regulation of nitrate assimilation and signaling and of plant growth remain enigmatic (7, 18). Among the TFs, direct interactions of *NLP6/7*,

*TGA1*, *bZIP1*, and *TCP20* with target gene promoters have been verified. No protein–protein interactions involved in nitrate signaling have yet been reported.

*TCP20* and *NLP6&7* belong to two ancient gene families, the protein sequences of which contain multiple, deeply conserved motifs in plants (19, 20). *NLP6&7* proteins are expressed in almost all organs (21–23). *NLP7* is an important regulator of PNR (10, 23). Nitrate does not induce *NLP7* mRNA but instead induces a rapid accumulation of *NLP7* in the nucleus by nuclear retention, leading to induction of nitrate-regulated genes (10). Transcriptomic analysis revealed that *NLP7* binds to and regulates 91 nitrate-regulated genes (10). In another study, the synthetic transcription activator, *NLP6-VP16*, was found to promote the expressions of nitrate-inducible genes; and the synthetic transcription repressor, *NLP6-SUPRD*, was found to suppress the expressions of nitrate-inducible genes (9). However, no phenotypes have been described for *NLP6* single mutants so its regulatory roles and functional relationship with *NLP7* have not been fully elucidated, and no *nlp6nlp7* double mutants have been reported. Interestingly, *NLPs* carry PB1 domains at their C termini, which are protein–protein interaction domains conserved in animals, fungi, amoebas, and plants and which are involved in responses to nutrients, growth factors, and stress (20, 24, 25). *TCP20* is a member of the class I TCP gene family (19). Throughout developmental stages, *TCP20* protein is expressed in embryos, seedlings, leaves, flower buds, and roots (22, 26, 27) and was found to regulate pavement cell size during early leaf development and onset of leaf senescence by promoting jasmonic acid synthesis. Although *TCP20* has no obvious role in PNR (16), it was found to bind to the DNA of over 100 nitrate-regulated genes (6, 26, 27), and it is the only gene identified in both local and

## Significance

Nitrate is an essential nutrient and a critical signal for plant growth, development, and stress responses. Nitrate signaling underlies a myriad of physiological, morphological, and developmental processes. Here we report that interacting teosinte branched1/cycloidea/proliferating cell factor and NIN-like protein transcription factors constitute a molecular link between nitrate signaling and the control of the cell-cycle progression gene *CYCB1;1* and root meristem growth. Our findings shed light on the regulatory mechanisms underlying an important plant adaptive process for coping with and surviving environmental challenges.

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<sup>1</sup>Present address: The Climate Corporation, St. Louis, MO 63141.

<sup>2</sup>To whom correspondence should be addressed. Email: nrcrawford@ucsd.edu.

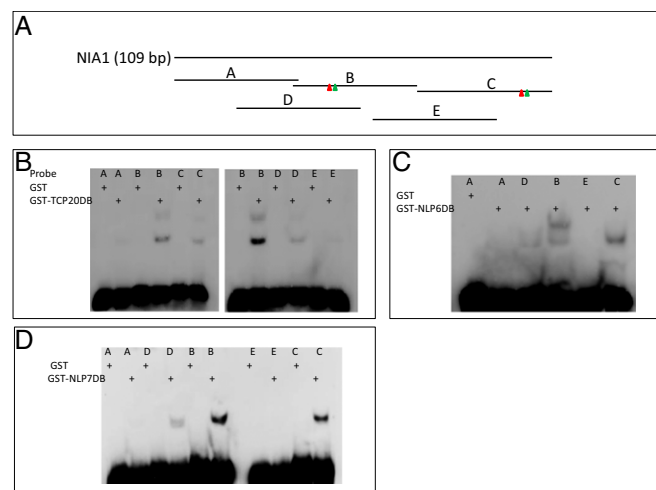
This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1615676114/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1615676114/-DCSupplemental).

systemic regulations of N root foraging (7, 16). In plant defense, TCP20 was recently identified among the TCP factors that are targets of pathogenic effectors (28). TCP20's DNA-binding properties also suggest its regulatory role in cell division, expansion, and differentiation (29, 30). In particular, TCP20 was reported to regulate mitotic cyclin gene *CYCBI;1* and ribosomal protein genes *in vivo* (30). *CYCBI;1* is an effector of growth control at the G<sub>2</sub>-to-M phase of the cell cycle. It is a marker of mitotic activity, particularly being a division marker of apical meristems, and also a DNA stress marker as an index of G<sub>2</sub>/M checkpoint arrest (31, 32).

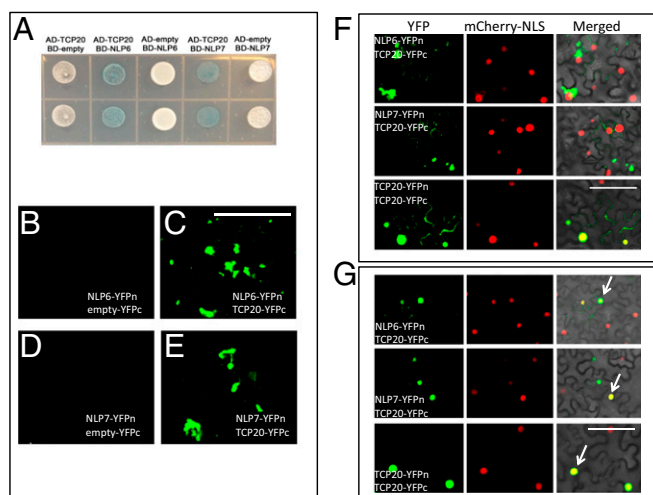
In this paper, we demonstrate that TCP20 and NLP6&7 physically interact under continuous nitrate and N-starvation conditions, forming heterodimers in different compartments of the cell. These interacting regulators were found to play an important role in controlling the expression of key nitrate-responsive genes and the G<sub>2</sub>/M cell-cycle marker gene, *CYCBI;1*. NLP6, like NLP7, is retained in the nucleus in the presence of nitrate, and, based on single- and double-mutant analyses, serves as a partially redundant activator along with NLP7. Under N starvation, TCP20-NLP6&7 complexes accumulate in the nucleus, which coincides with TCP20, NLP6&7-dependent regulation of nitrate assimilation and signaling genes, of the cell-cycle progression gene *CYCBI;1*, and of root meristem growth.

## Results

**The Binding Sites of TCP20, NLP6, and NLP7 on a 109-bp NIA1 Enhancer Fragment Are in Close Proximity to Each Other.** In our search for direct nitrate regulators, a 109-bp *cis* regulatory module was identified as nitrate enhancer in the *NIA1* promoter (33). The 109-bp *NIA1* enhancer fragment was incorporated into a nitrate-inducible reporter construct-YFP and used in a forward genetic screen, which yielded mutations in *NRT1.1*, a nitrate transporter (34, 35), and *NLP7* (34). The enhancer fragment was also used in yeast one-hybrid screens, which identified *TCP20* (16) and *NLP6*. In this study, we first examined TCP20, NLP6, and NLP7 binding to the 109-bp *NIA1* enhancer fragment using electrophoretic mobility shift assays (EMSA). The DNA-binding domains of all three proteins (TCP20-DB, NLP6-DB, and NLP7-DB) bound to the same subfragments of the 109-bp *NIA1* enhancer fragment (Fig. 1). Furthermore, a set of mutant probes



**Fig. 1.** Binding of TCP20 DNA-binding domain (DB), NLP6-DB, and NLP7-DB to the fragments of *NIA1* (109 bp) DNA was determined by EMSA. (A) Diagram of probes. Red and green triangles represent TCP20 (red) and NLP6/7 (green) binding sites, respectively. (B–D) Binding of TCP20DB, NLP6DB, and NLP7DB to A, B, C, D, and E probes. The probes are listed in *SI Appendix, Table S1*.



**Fig. 2.** Protein–protein interactions among TCP20 and NLP6&7. (A) Blue and white colonies in yeast two-hybrid assay for testing interaction between TCP20 and NLP6 and TCP20 and NLP7. (B–E) BiFC in *N. benthamiana*. (F and G) The subcellular locations of TCP20–NLP6/NLP7 interactions are affected by N status. Red mCherry was used as a marker for nuclei. BiFC was conducted in *N. benthamiana* grown on 5 mM KNO<sub>3</sub> as the sole N source (F) or the plants grown on 5 mM KNO<sub>3</sub> and then grown on N-free medium for 4 days (G, 0 mM KNO<sub>3</sub>). White arrow indicates nuclear localization. Because the nuclear marker (mCherry-NLS) and the NLP marker (NLP-YFP) are carried on separate plasmids, overlapping signals (yellow) occur only in cells that have been transformed with both plasmids. (Scale bars: 100 μm.)

was used to identify DNA-specific *cis*-elements that interact with these three proteins (*SI Appendix, Fig. S1* and *Table S1*). We found that the TCP20 and NLP6&7 sites either overlap or are in close proximity within two subfragments (probes B and C) (Fig. 1 and *SI Appendix, Fig. S1*). Interestingly, in our previous study of nitrate regulatory elements in the 109-bp *NIA1* enhancer fragment, the CGCCACT sequence, which overlaps TCP20-NLP6&7 binding sites in probe C, was found to be critical for nitrate induction (19), whereas none of these nitrate-responsive elements was identified in probe B (19). In addition, the close proximity of the TCP20 and NLP6&7 binding sites suggests that each NLP might interact with TCP20 when binding to the 109-bp fragment.

## TCP20, NLP6, and NLP7 Physically Interact with Each Other and Subcellular Localizations of Their Interactions Depend on Nitrate Availability.

We next tested for protein interactions using both yeast two-hybrid (Fig. 2A) and bimolecular fluorescence complementation (BiFC) assays (Fig. 2B). In the yeast two-hybrid assays, both NLP6 and NLP7 strongly interact with TCP20 as seen by the blue yeast colonies (Fig. 2A). These interactions were further confirmed by BiFC assays in *Nicotiana benthamiana* by agro-infiltration, which showed green fluorescence indicative of heterodimer formation (Fig. 2B–E; the mCherry-NLS was used as a marker for nuclei). The BiFC constructs of NLP6&7-nYFPs and TCP20-cYFPs were transformed and tested in transgenic *Arabidopsis* plants. The results in transgenic *Arabidopsis* plants validated the presence of the TCP20-NLPs interactions and showed that the interactions occurred in roots (*SI Appendix, Fig. S2*). We also examined self-interactions for TCP20 (*SI Appendix, Fig. S3*), NLP6, and NLP7 (*SI Appendix, Fig. S4 A–C*). TCP20 has been reported to be a dimeric protein located mainly in the nucleus (27). Our data confirm this finding in that TCP20-YFPn and TCP20-YFPc indeed interact, primarily in the nucleus and but in other locations as well. Additionally, NLP6 and NLP7 each interact with themselves and with each other (*SI Appendix, Fig. S4*).

We next tested the effects of N growth conditions on the subcellular localizations of the TCP20 and NLP6&7 proteins and of

the TCP20-NLP heterodimers. First, cellular localizations were examined under two treatment conditions: continuous nitrate (no ammonium) (*SI Appendix, Fig. S5 A–C*) and N starvation (after growth on  $\text{KNO}_3$ ) (*SI Appendix, Fig. S5 D–F*). Each protein was fused to YFP and then transiently expressed in *N. benthamiana* by using *Agrobacterium* with DNA constructs driven by the CaMV 35S promoter. TCP20 was found primarily in the nucleus under both conditions (*SI Appendix, Fig. S5 C and F*), consistent with a previous report for transgenic *Arabidopsis* plants (27). Nuclear retention was also found for NLP6 and NLP7 in nitrate-grown plants with some additional signal outside the nucleus (*SI Appendix, Fig. S5 A and B*). Under N starvation, signals for NLP6 and NLP7 were very low. A few NLP6 or 7-YFP fusion proteins were found in the nucleus, whereas a majority of the signals were found outside the nucleus (*SI Appendix, Fig. S5 D and E*). We validated the findings in the roots of NLP6/7-YFP transgenic plants. (*SI Appendix, Fig. S5 G–L*). Our findings are consistent with those of Marchive et al. (10) who showed nuclear retention of NLP7 in nitrate-treated plants. In addition, our data indicate that NLP6, like NLP7, is retained in the nucleus in the presence of nitrate.

Next, the effect of N growth conditions on TCP20-NLP interactions was tested. Under both conditions (continuous nitrate versus N starvation), NLP6 and NLP7 interacted with TCP20 in the BiFC experiments (Fig. 2 *F* and *G* and *SI Appendix, Fig. S6*); however, the primary locations of NLP6&7-TCP20 heterodimers varied dramatically. Under nitrate-grown conditions, NLP6&7-TCP20 heterodimers reside primarily outside the nucleus (Fig. 2*F* and *SI Appendix, Fig. S6*), whereas under N starvation, the heterodimers are primarily located in the nucleus (Fig. 2*G*). We validated the findings in transgenic *Arabidopsis* roots (*SI Appendix, Fig. S7*). This is the opposite of what was found for the NLP single proteins (i.e., NLP6&7-YFP fusions) (*SI Appendix, Fig. S5*) (10). The TCP20 homodimers, however, were found primarily in the nucleus under both conditions with additional signal outside the nucleus in nitrate-grown plants (Fig. 2*F* and *G*). NLP6 and NLP7 homodimers and heterodimers were found outside the nucleus in the presence of nitrate (*SI Appendix, Fig. S4*), whereas no signal was detected in N-starved plants (*SI Appendix, Fig. S8*). These results suggest that NLP 6&7 homodimers and heterodimers are not functioning as the active complexes in nuclei to induce gene expression in the presence of nitrate.

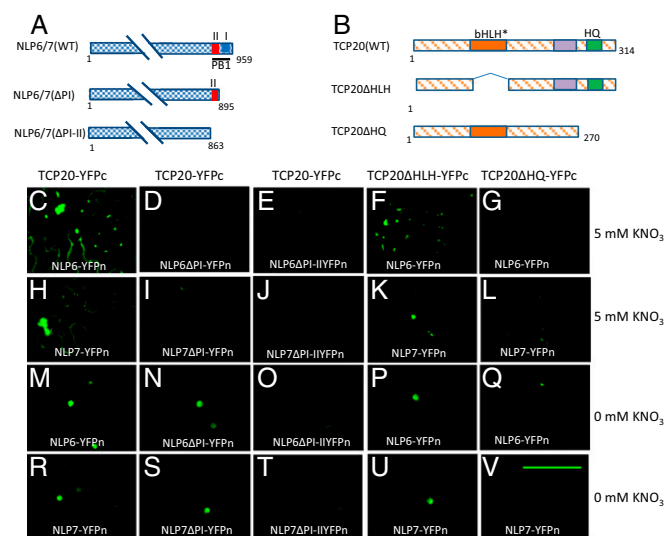
**The Type I/II PB1 Domains of NLP6&7 and the Glutamine-Rich Domain of TCP20 Are Required for Protein-Protein Interactions.** We also investigated potential interaction domains in NLP6&7 and TCP20. The NLP6&7 PB1 domains display both type I and type II structures containing key signature residues: a conserved Lys residue in the type II structure and an OPCA [the octicosapeptide repeat (OPR), p40phox and budding yeast Cdc24p (PC), and atypical protein kinase C-interaction domain (AID)] motif in the type I structure (*SI Appendix, Fig. S9A*). As an example, the type I/II PB1 domain is present in the mammalian scaffold protein p62, a crucial regulator linking amino acids to the activation of the mammalian target of rapamycin complex 1 (mTORC1) to control cell size and proliferation (24, 36, 37). TCP20 contains a basic helix-loop-helix-like motif (bHLH\*) that is involved in dimerization (38). We also identified two other candidate domains in the TCP20 C terminus. One is a histidine- and glutamine-rich (HQ-rich); the other is next to the HQ-rich domain and within a larger glycine-rich domain (*SI Appendix, Fig. S9B*). Both of these domains contain residues that are deeply conserved across plant species. We hypothesized that these domains could be involved in the TCP20-NLP6&7 protein interactions.

To determine the function of these domains, various deleted derivatives of TCP20 and NLP6&7 were constructed (Fig. 3 *A* and *B*) and then examined in BiFC assays. Deletion constructs of NLP6&7 were tested first. Deleting the OPCA motif ( $\Delta$ PI) of NLP6 and NLP7 (leaving the first lysine-region intact) or both regions of PB1 ( $\Delta$ PI-II) abolished the TCP20-NLP6&7 interactions

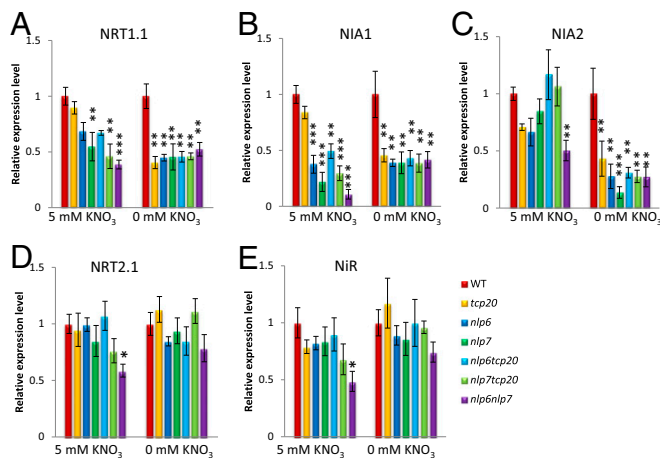
under 5 mM  $\text{KNO}_3$  growth conditions (Fig. 3 *D, E, I, and J*). Interestingly, under N-starvation conditions, TCP20-NLP6&7 interactions were abolished only when both PB1 motifs ( $\Delta$ PI-II) were deleted (Fig. 3 *O* and *T*) because interaction was still evident in the single OPCA motif deletion construct ( $\Delta$ PI) (Fig. 3 *N* and *S*). For NLP6&7 homodimer interactions, deletion of the OPCA region alone ( $\Delta$ PI) or both regions of PB1 ( $\Delta$ PI-II) abolished the interactions (*SI Appendix, Fig. S10*). These results are consistent with previous findings showing that type I/II PB1 domains are critical for the formation of homo- and heterodimers and in interactions with proteins lacking PB1 domains (24, 25). Next, deletion constructs of TCP20 were analyzed. Deletion of the N terminus of TCP20 (TCP20 $\Delta$ 1–78) led to a small decrease in the TCP20-NLP6&7 interactions under nitrate growth or N starvation (*SI Appendix, Fig. S11*). Deletion of bHLH-like motif in TCP20 (TCP20 $\Delta$ HLH; Fig. 3 *F, K, P, and U*) had no impact on the interactions under both conditions. However, deleting the amino acids from 270 to 314 (TCP20 $\Delta$ HQ) in the C-terminal domain of TCP20 significantly decreased or abolished the TCP20-NLP6&7 interactions under both nitrate-grown (Fig. 3 *G* and *L*) and N starvation (Fig. 3 *Q* and *V*) conditions. Further deletion of the other conserved region (TCP20 $\Delta$ 230–314) showed the same results (*SI Appendix, Fig. S11*). Overall, these results indicate that both the PB1 domains of NLP6&7 and the C-terminal HQ-rich domain of TCP20 are necessary for the TCP20-NLP6&7 interactions. The N terminus and bHLH-like domain of TCP20 are dispensable.

#### Analysis of *tcp20*, *nlp6*, and *nlp7* Single and Double Mutants Reveals Distinctive Regulatory Roles of the TFs in Nitrate Assimilation and Signaling Under Continuous Nitrate and N-Starvation Conditions.

To dissect individual and combinatorial molecular functions of NLP6, NLP7, and TCP20 on expression of genes involved in nitrate transport, assimilation, and signaling, we conducted quantitative RT-PCR on whole roots of wild-type (WT) and single- and double-mutant lines under continuous nitrate-grown



**Fig. 3.** PB1 domain of NLP6/7 and a C-terminal HQ region of TCP20 are required for TCP20-NLP6/7 interaction. (*A* and *B*) Diagrams of NLP6/7 and TCP20 derivatives. The numbers shown for NLP6/7 are for NLP7. Corresponding number for NLP6 are the following: full length, 841 amino acids; deletion derivatives, 741 or 774 amino acids. For NLPs, red and dark-blue boxes indicate two modules (II and I) in PB1 domain. For TCP20, orange and green boxes present bHLH\* and HQ, respectively. Purple box presents a conserved region next to HQ. (*C–V*) BiFC was conducted in leaves of *N. benthamiana* under 5 mM  $\text{KNO}_3$  growth condition (*C–L*) or the plants grown on 5 mM  $\text{KNO}_3$  and then grown on N-free medium for 4 days (*M–V*). (Scale bar: 100  $\mu\text{m}$ .)



**Fig. 4.** The interacting transcriptional factors, TCP20 and NLP6&7, regulate key genes of nitrate assimilation and signaling in response to nitrate status. (A–E) RT-PCR quantification of *NRT1.1*, *NIA1*, *NIA2*, *NRT2.1*, and *NiR* under nitrate-grown condition and N starvation. WT mRNA level was set to 1 for each condition. Error bars show SEM ( $n = 3$  biological replicates). *Arabidopsis* seedlings were grown on 5-mM  $\text{KNO}_3$  plates for 6–7 days and then were transferred to new 5 mM  $\text{KNO}_3$  plates or N-free plates for 3 d. Total roots were harvested for analysis. For each experiment, within each nitrate condition, one-way ANOVA was performed and followed by a  $t$  test (two-side, using WT as control). Black asterisks represent statistical difference between WT and mutants under same nitrate condition.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ . (Scale bar: 50  $\mu\text{m}$ .)

and N-starvation conditions (Fig. 4). First, *nlp6* and *nlp7* single mutants showed lower levels of *NRT1.1*, *NIA1*, and *NIA2* mRNAs under nitrate-grown conditions (Fig. 4 A–C); levels of *NRT2.1* and *NiR* mRNAs, however, were similar to WT under these conditions (Fig. 4 D and E). *nlp6nlp7* double mutants showed significant reductions in mRNA levels compared with WT across five target genes as follows: *NIA1* (~90%), *NIA2* (~50%), *NRT1.1* (~61%), *NiR* (~50%), and *NRT2.1* (~40%) (Fig. 4). Compared with *nlp6* and *nlp7* single mutants, the further significant reductions of mRNA levels in *nlp6nlp7* double mutants were observed across all of the target genes compared with at least one of the single mutants (Fig. 4 A–E and *SI Appendix*, Fig. S12). These are phenotypes of *nlp6* single and *nlp6nlp7* double mutants. The results suggest that NLP6 and NLP7 are partially redundant activators of key nitrate transport, assimilation, and signaling genes in the presence of nitrate and function additively. Consistent with this proposal, *nlp6nlp7* double mutants showed severe growth defects with nitrate as the sole N source but not with ammonium (*SI Appendix*, Fig. S13), which is similar to the propagation and growth properties observed in NR-null (*nia1nia2*) mutants (6). Next, *tcp20* single and *tcp20nlp6* and *tcp20nlp7* double mutants were examined under nitrate-grown conditions. The *tcp20* single mutation had little effect on mRNA levels across the target genes (Fig. 4), which is consistent with what was reported in a previous study (30). The *tcp20nlp6* and *tcp20nlp7* double mutants displayed no obvious additive effects (Fig. 4).

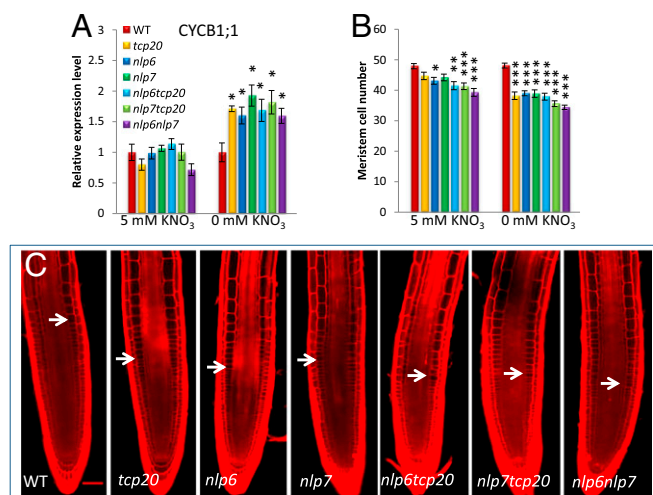
Under N starvation, however, the mRNA levels of *NRT1.1*, *NIA1*, and *NIA2* were significantly and uniformly lower across all of the mutant lines (~50–60% reduction) (Fig. 4 A–C). However, no evident reduction in mRNA levels of *NRT2.1* and *NiR* was observed (Fig. 4 D and E). Especially interesting was the effect of *tcp20* mutations, which showed strong reductions in mRNA levels of *NRT1.1*, *NIA1*, and *NIA2*, similar to what was observed for *nlp6nlp7* double mutants (Fig. 4 A–C). This contrasts to the lack of effect of TCP20 in the presence of nitrate. These results are consistent with the nuclear TCP20–NLP6&7 interactions under N starvation (Fig. 2G), suggesting that these proteins may function as a complex in regulating expression of *NRT1.1*, *NIA1*,

and *NIA2* under N starvation. In addition to strongly regulating NR genes, the interacting transcriptional regulators also control the mRNA levels of *NRT1.1* (*CHL1/NPF6.3*), which plays a versatile and central role in adaptive responses to nitrate availability (35, 39, 40).

A two-way ANOVA analysis revealed that both main effects of N conditions and genotypes are statistically significant in regulating expression of *NRT1.1* ( $***P < 0.001$ ), *NIA1* ( $***P < 0.001$ ), and *NIA2* ( $***P < 0.001$ ), and an interaction effect between N treatment and genotypes is also statistically significant in regulating expression of *NRT1.1* ( $*P < 0.05$ ), *NIA1* ( $*P < 0.05$ ), and *NIA2* ( $***P < 0.001$ ) (*SI Appendix*, Table S4). These results demonstrate that these interacting regulators control the expression of the key genes *NRT1.1*, *NIA1*, and *NIA2* and that the regulatory controls are distinct between nitrate-grown conditions and N starvation. The expression of *NRT1.1*, *NIA1*, and *NIA2* genes under N starvation corresponds to nuclear localization of the interacting transcriptional regulators and supports the idea that these regulators are working in concert to promote the expression of these genes under N starvation.

#### Interacting TCP20 and NLP6&7 Are Required for Proper Expression of the Cell-Cycle Progression Gene *CYCBI;1* and Root Meristem Growth Under N Starvation.

We next addressed the question, What additional regulatory roles do TCP20 and NLP6&7 play in response to nitrate availability beyond regulating the nitrate assimilation and signaling genes? TCP20 is known to bind to the GCCCR motif in the promoter of mitotic cyclin gene *CYCBI;1* in vitro and in vivo (30). We further examined the *CYCBI;1* promoter region containing the GCCCR motif, which, interestingly, led to the identification of two potential NLP6&7-binding sites (GCCCACTT and CGGGCCTT) in the same region (*SI Appendix*, Fig. S14). The two consensus sequences only have a two-nucleotide mismatch compared with the



**Fig. 5.** The interacting transcriptional factors TCP20 and NLP6&7 regulate the cell-cycle gene *CYCBI;1* and control cell division in the root meristem in response to nitrate status. (A) *CYCBI;1* gene expression level from total roots in WT and mutant lines (conditions here are the same as those of A–C in Fig. 4). Error bars show SEM ( $n = 3$  biological replicates). (B) Meristem cell number in WT and mutant lines under 5 mM  $\text{KNO}_3$  growth condition and N starvation for 3 d. Root meristem number was measured by counting the cortical cells from the quiescent center to the first cell that clearly doubles its volume. Error bars represent SEM ( $n = 18$ –20). (C) Confocal images of WT, *tcp20*, *nlp6*, *nlp7*, *nlp6tcp20*, *nlp7tcp20*, and *nlp6nlp7* root tips stained with propidium iodide. White arrows indicate the boundary of the apical meristem region. Seedlings were grown on 5 mM  $\text{KNO}_3$  medium for 3 d and then were transferred to N-free medium for 3 d. The same statistical test used in Fig. 4 has been used here.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ . (Scale bar: 50  $\mu\text{m}$ .)

sequences of the 109-bp *NLA1* promoter fragment (*SI Appendix, Fig. S1*), and each contains a GCCCR motif recognized by TCP20 (*SI Appendix, Fig. S14*). In addition, in close proximity to the putative NLP6&7-binding sites were another two GCCCR motifs and an auxin response factor (ARF)-binding site (*SI Appendix, Fig. S14*) (30). As shown in Fig. 5A, under nitrate-grown conditions there was some reduction of *CYCB1;1* mRNA levels measured in whole roots of the *nlp6nlp7* double- and *tcp20* single-mutant lines, but neither was statistically significant. No reduction in other mutant lines was observed. In contrast, under N starvation, the mRNA levels of *CYCB1;1* measured in whole roots were significantly and uniformly higher across all of the mutant lines (Fig. 5A). A two-way ANOVA analysis revealed that, in regulating expression of *CYCB1;1*, both main effects of N conditions and genotypes are statistically significant ( $***P < 0.001$ ;  $*P < 0.05$ ), and an interaction effect between N treatment and genotypes is also statistically significant ( $*P < 0.05$ ) (*SI Appendix, Table S4*). These results also indicate that the regulation of this key cyclin gene by the interacting nitrate regulators is distinct between nitrate-grown conditions and N starvation. The close proximity of the confirmed binding sites of TCP20 and NLP6&7 in the *NLA1* promoter fragment (Fig. 1 and *SI Appendix, Fig. S1*) and their putative binding sites found in the *CYCB1;1* promoter region (*SI Appendix, Fig. S14*) suggest that the interaction of these regulators might play a role in responses to nitrate availability for both nitrate assimilation and signaling and cyclin genes.

To investigate further the linkage between nitrate regulation and growth via cell-cycle control, primary root growth phenotypes in the single and double mutants of *TCP20* and *NLP6&7* were examined (*SI Appendix, Fig. S15*). Overall, all of the mutants had shorter roots than the WT. The mutants under N starvation displayed significantly greater reductions in primary root length than the mutants under nitrate-grown conditions (*SI Appendix, Fig. S15*). In addition, under nitrate-grown conditions, *nlp6nlp7* double mutants showed stronger deleterious effects on primary root length than the single mutants (*SI Appendix, Fig. S15 A and C*), consistent with our previous finding in the mRNA levels of nitrate sentinel genes. Meristem size and cell numbers, which are controlled by cell division and are a determinant of root growth, were then examined in primary roots under two distinct nitrate conditions. All single and double mutants showed reduced levels of cell numbers and meristem length (Fig. 5B and C; *SI Appendix, Fig. S15D*). The phenotypes were more conspicuous under N starvation than those under nitrate-grown conditions (Fig. 5B and C; *SI Appendix, Fig. S15D*). A two-way ANOVA analysis revealed that an interaction effect between N treatment and genotypes is statistically significant in regulating these growth phenotypes: primary root meristem cell number and length ( $*P \leq 0.05$ ) and primary root length ( $***P < 0.001$ ); and both main effects of N conditions and genotypes are statistically significant ( $***P < 0.001$ ) (*SI Appendix, Table S4*). The mutation effects on meristem size and cell numbers are largely consistent with what we observed in primary root length, supporting the idea that there is a direct correlation between nitrate-regulated *CYCB1;1* levels and primary root growth. The higher mRNA levels of *CYCB1;1* under N starvation in the mutants could result in one of two outcomes: increased cell production rate in apical meristem or arrest at G2/M checkpoint in cell cycle as has been observed under stress conditions (32, 41). The elevated mRNA levels of *CYCB1;1* coincided with the decreased number of cells in the root apical meristem in our experiments, supporting G2/M cell-cycle arrest as the reason for the observed effects.

## Discussion

We have demonstrated that TCP20 physically interacts with both NLP6 and NLP7 under nitrate-grown and N-starvation conditions

and that both the type I/II PB1 domain of NLP6&7 and the histidine- and glutamine-rich domain of TCP20 are necessary for these interactions. Under N starvation, TCP20-NLP6&7 heterodimers accumulate in the nuclei, which correlates with the regulation of nitrate assimilation and signaling genes and the mitotic cyclin gene *CYCB1;1* to counteract N-starvation stress. Mutants show significantly stunted root growth and decreased root meristem size and meristem cell numbers, which we suggest is a result of premature cell-cycle exit due to the misregulation of *CYCB1;1*. In addition, NLP6 and NLP7 physically interact with each other and themselves via the PB1 domain and are partially redundant activators in the presence of nitrate. These results suggest that these three transcription factors form a regulatory nexus integrating the regulation of PNR genes in the presence and the absence of nitrate and linking nitrate responses to root growth.

The regulatory roles of AtTCP20 in response to nitrate availability compare with those of AtTCP21/CHE (for CCA1 hiking expedition) in the circadian oscillator (42) in that both these class I TCP genes are crucial for plant responses to variations in environmental cues, i.e., nutrients, light, and temperature. The type I/II PB1 domains that we found are required for forming TCP20-NLP6&7 and NLP6&7 self/hetero-complexes are also found to be important for other key interactions. They are needed for the homo- and hetero-oligomerization of auxin response factor (ARF) transcription factors and auxin/indole 3-acetic acid (Aux/IAA) repressor proteins (25, 43) as well as for organizing growth factor and nutrient signaling within the mTORC1 pathway in animals (37). Thus, the type of interactions uncovered for TCP20, NLP6, and NLP7 regulators are part of a more general pattern used for signaling in both plants and animals.

In plants, growing evidence indicates that complex crosstalk of nitrate and hormone signaling, and a close connection between TCP regulation and hormone signaling, underlie the integrated control of plant growth, development, and defense response (7, 44, 45). Nitrate controls hormonal pathways in biosynthesis, transport, and signal transduction, and, conversely, hormonal signaling regulates nitrate/nitrogen transport and assimilation to adjust nutritional status to growth (7, 44). On the other hand, in addition to acting as transcriptional modulators of cell division, TCPs affect hormone synthesis, transport, and signal transduction by controlling gene expression through their context-dependent interaction with tissue-specific cellular components (45). Our study reveals that TCP regulation is part of nitrate signaling, providing a transcriptional control of cell-cycle genes when plants face loss of nitrate availability. Our findings offer insight into the close interplays and convergent regulations between nitrate- and hormone-signaling pathways in plants. It will be interesting to investigate in the future whether PB1 domain-dependent signaling complexes may provide additional links between nitrate- and hormone-signaling pathways and whether other members of the TCP family function in a similar fashion as TCP20 in nitrate signaling.

## Materials and Methods

Details of plant material and growth conditions, plasmid constructions, protein expression and purification, EMSA, yeast two-hybrids, BiFC and transient expression, transgenic lines, confocal microscopy, and quantitative PCR are given in *SI Appendix, Materials and Methods*.

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