Discovery of nitrate–CPK–NLP signalling in central nutrient–growth networks

Kun-hsiang Liu1,2, Yajie Niu1, Mineko Konishi3, Yue Wu1, Hao Du1, Hoo Sun Chung1, Lei Li1, Marie Boudsocq1,4, Matthew McCormack1, Shugo Maekawa3, Tetsuya Ishida3, Chao Zhang5, Kevan Shokat5, Shuichi Yanagisawa3 & Jen Sheen1

Nutrient signalling integrates and coordinates gene expression, metabolism and growth. However, its primary molecular mechanisms remain incompletely understood in plants and animals. Here we report unique Ca2+ signalling triggered by nitrate with live imaging of an ultrasensitive biosensor in Arabidopsis leaves and roots. A nitrate-sensitized and targeted functional genomic screen identifies subgroup III Ca2+-sensor protein kinases (CPKs) as master regulators that orchestrate primary nitrate responses. A chemical switch with the engineered mutant CPK10(M141G) circumvents embryo lethality and enables conditional analyses of cpk10 cpk30 cpk32 triple mutants to define comprehensive nitrate–associated regulatory and developmental programs. Nitrate–coupled CPK signalling phosphorylates NIN–LIKE PROTEIN (NLP) transcription factors to specify the reprogramming of gene sets for downstream transcription factors, transporters, nitrogen assimilation, carbon/nitrogen metabolism, redox, signalling, hormones and proliferation. Conditional cpk10 cpk30 cpk32 and nlp7 mutants similarly impair nitrate–stimulated system-wide shoot growth and root establishment. The nutrient–coupled Ca2+ signalling network integrates transcriptional and cellular metabolism with shoot–root coordination and developmental plasticity in shaping organ biomass and architecture.

Nitrate triggers unique Ca2+-CPK signalling

To determine the primary mechanism in nitrate signalling, we developed integrated seedling and cell-based assays in Arabidopsis. Without exogenous nitrogen, seed reserves supported rapid initial seedling growth after germination (Fig. 1b). However, the increase in biomass ceased after four days even with illumination and sucrose supplies (Fig. 1c, d). Although ammonium (NH4+ or glutamine (Gln) was transported, sensed and used2–21, only nitrate (0.1–10 mM) promoted distinct shoot biomass accumulation and root system architecture establishment. Nitrate, but not metabolites, NH4+ or Gln that are derived from nitrate assimilation, sustained substantial postembryonic growth (Fig. 1a, c, d and Extended Data Fig. 1a), consistent with uncoupling of nitrate signalling from nitrate metabolism2–3, NH4+ or Gln signalling2,30,31.

Although nitrate-triggered Ca2+ signals could be detected using transgenic aequorin seedlings22,29, the response was subtle when compared with the flg22-induced Ca2+ signals in immune signalling (Extended Data Fig. 1b, c). As nitrate signalling and assimilation occur in both leaves and roots (Extended Data Fig. 1a, d, e), we developed a versatile single-cell system to investigate live Ca2+ signalling stimulated by nitrate in mesophyll protoplasts22,23,32. In leaf cells co-expressing the ultrasensitive Ca2+ biosensor GCaMP6 (ref. 33) and a nuclear mCherry, single-cell live recordings revealed that nitrate specifically stimulated a unique and dynamic Ca2+ signature in the nucleus and cytosol (Fig. 1e, f). Chelating exogenous Ca2+ by EGTA diminished the nitrate-triggered Ca2+ signature (Fig. 1f). Unlike the transient and acute cytosolic calcium concentration ([Ca2+]cyt) increase stimulated by osmotic and cold stresses in seedlings22,34, nuclear Ca2+ oscillation in symbiosis35, or a 10-s [Ca2+]cyt peak triggered by nitrate in roots29, a gradually rising subcellular Ca2+ signature

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

1Department of Molecular Biology and Centre for Computational and Integrative Biology, Massachusetts General Hospital, and Department of Genetics, Harvard Medical School, Boston, Massachusetts 02114, USA. 2Basic Forestry and Proteomics Research Center, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China. 3Biotechnology Research Center, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan. 4Institute of Plant Sciences Paris-Saclay (IPS2), CNRS, INRA, Université Paris-Sud, Université d’Evry Val d’Essonne, Université Paris-Diderot, Sorbonne Paris-Cité, Université Paris-Saclay, Bâtiment 630, 91405 Orsay, France. 5Howard Hughes Medical Institute and Department of Cellular and Molecular Pharmacology, UCSF, 600 16th St, San Francisco, California 94143, USA.

*These authors contributed equally to this work.
apparented to nitrate over a course of minutes was recorded by GCaMP6-m fluorescent intensity. Error bars (grey areas, 199 error bars) denote s.e.m., F = 0 denotes the relative fluorescence intensity. Error bars (grey areas, 199 error bars) denote s.e.m., n = 10 protoplasts. g. Time-lapse images of Ca2+ signalling in mesophyll cells of cotyledons in GCaMP6 transgenic plants. 10 mM KNO3. BF, bright field. NLS–mCh denotes nuclear localization. Scale bars, 10 μm. Images are representative of 10 cotyledons. h. Subgroup III CPKac – Flag expression was determined by immunoblot analyses. Source Data can be found in the Supplementary Information. i. The relation tree illustrating the family connections of subgroup III CPKs.

To search for candidates of intracellular Ca2+ sensors mediating primary nitrate signalling, we conducted an in-gel kinase assay and detected enhanced activity of endogenous CPKs in response to nitrate within 10 min (Extended Data Fig. 2c). The molecular mass of nitrate-activated protein kinases was similar to that of most CPKs but not CIPKs17–19,23. There are 34 Arabidopsis CPK genes mediating diverse signalling pathways with complex and redundant functions23,24,26,36. As cpk mutants have escaped from mutant screens for nitrate signalling,7,11, a simple, rapid and reliable cell-based reporter assay could facilitate targeted functional genomic screening of CPK candidates involved in nitrate signalling21. Using a luciferase (LUC) reporter gene NIR-LUC that exhibits a physiological nitrate response in transgenic Arabidopsis plants8,1, we observed rapid, specific and sensitive regulation of LUC activity by 0.1–50 mM KNO3 within 2 h (Extended Data Fig. 2d–f). Unexpectedly, co-expressing NIR-LUC with a constitutively active construct (CPKac)23 for each of the 25 CPK genes expressed in mesophyll protoplasts did not trigger marked NIR-LUC activation (Fig. 1h). To test the possibility that the CPKac-mediated response could be sensitized by low nitrate, we added 0.5 mM KNO3 to the protoplast incubation medium. Interestingly, subgroup III constitutively active CPKs (CPK7ac, CPK8ac, CPK10ac, CPK13ac, CPK30ac and CPK32ac) were most effective at synergistically activating NIR-LUC together with 0.5 mM KNO3 (Fig. 1h, i and Extended Data Fig. 2g). Although specific CPKac is sufficient to activate stress or immune responses associated with strong and distinct Ca2+ signalling patterns22–24,26,34, activation of subgroup III CPKac alone did not fully evoke specific nitrate response. Consistent with our findings, it has been suggested that primary nitrate signalling requires the coordination of multiple signalling mechanisms26,7,11,12,13,18,37. Our results indicate that nitrate may specify and synergize functionally redundant subgroup III CPKs in NIR-LUC activation.

Chemical switch defines CPK signalling

To test the specificity and physiological roles of subgroup III CPKs, we examined loss-of-function cpk mutants. The single cpk7, cpk8, cpk10, cpk13, cpk30 or cpk32 mutants barely affected nitrate-responsive genes and lacked overt growth phenotypes (Extended Data Fig. 3a–g). As CPK10 and CPK30 share the highest sequence identity and slight modulation of NIR and G6PD3 expression (Fig. 1i and Extended Data Figs 2g, 3d), we attempted to generate the cpk10 cpk30 double mutant. However, analyses of the siliques revealed that the cpk10 cpk30 double mutant was lethal at early embryogenesis (Fig. 2a, b).

Aiming to simultaneously overcome embryo lethality and enable analyses of higher-order cpk mutants, we engineered CPK10 to be reversibly inhibited by 3MBiP (1-isopropyl-3-(3-methylbenzyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine), a specific and improved derivative of the protein kinase inhibitor PPI (4-amino-5-(methylphene)-7-(t-butyl)pyrazolo-[3,4-d]pyridimidine)8,39 (Fig. 2c). A critical determinant of protein kinase inhibitor selectivity is the size of the gatekeeper residue in the ATP-binding pocket18,39. By aligning the conserved protein kinase subdomain V in Arabidopsis CPK10, CPK30 and human CaMKI10, we created the CPK10(M141G) gatekeeper residue mutant (Fig. 2d). Notably, 3MBiP completely inhibited the kinase activity of CPK10(M141G) but not wild-type CPK10 (Fig. 2e).

A genomic construct expressing CPK10(M141G) was introduced into cpk10 cpk30+ to rescue the lethal embryo phenotype of its cpk10 cpk30 progenies, creating a 3MBiP-inducible icpk10,30 mutant (Fig. 2a, b). The haemagglutinin (HA)-tagged CPK10(M141G) protein immunoprecipitated from the transgenic plants was completely inhibited by 3MBiP (Fig. 2f). Because CPK32ac greatly enhanced NIR-LUC expression (Fig. 1h), we also generated cpk10 cpk32 and cpk30 cpk32 double mutants, as well as a 3MBiP-inducible icpk10,30,32 triple mutant expressing CPK10(M141G)–HA (designated icpk) by genetic crosses and molecular confirmation. Nitrate-responsive marker genes were reduced in double mutants, such as cpk10 cpk32, cpk30 cpk32 and icpk10,30, in the presence of 3MBiP (Extended Data Fig. 3i, j).
Nitrate–CPKs dictate growth plasticity

To investigate the nexus between nitrate-specific developmental programs (Fig. 1c, d) and nitrate–CPK signalling, we examined shoot growth by quantifying shoot fresh weight in wild-type and icpk seedlings. To bypass any early effect of nitrate on seed germination, we selected 3-day-old seedlings without apparent phenotypes on ammonium plates and placed them on 3MBiP medium in the presence of

pathway, nitrate and amino acid transporters, nitrogen metabolism, carbohydrate metabolism, signalling, cytokinin synthesis, and transcription factors in wild-type and icpk mutant plants (Extended Data Fig. 4f, g and Supplementary Table 2). Notably, nitrate activation of CYP735A2 expression to enhance trans-zeatin synthesis is crucial for shoot development, providing an interconnection between local and systemic nitrate signalling via the action of a mobile growth hormone40.

As nitrate activation of these marker genes was markedly diminished in the icpk mutant, CPK10, CPK30 and CPK32 are master regulators in primary nitrate signalling and integrate global gene expression to nitrate-activated metabolic and physiological responses (Fig. 3, Extended Data Fig. 4e–g and Supplementary Table 2).

Nitrate–CPKs control primary transcription

To explore the genome-wide transcriptional landscape controlled by nitrate–CPK signalling, we conducted RNA sequencing (RNA-seq) experiments 15 min after nitrate induction in wild-type and icpk seedlings. In wild-type seedlings, 394 and 79 genes were activated and repressed, respectively, in response to nitrate (log2 ≥ 1 or ≤ −1; q ≤ 0.05), which significantly overlapped with nitrate-responsive genes discovered in microarray-based studies of both roots and shoots4,8,10,13.

In icpk, 266 upregulated (68%) and 44 downregulated (56%) genes could be defined as statistically significant nitrate–CPK target genes (icpk KNO3 versus wild-type KNO3, q ≤ 0.05) (Fig. 3, Extended Data Figs 4, 5 and Supplementary Tables 1 and 2). Thus, CPK10, CPK30 and CPK32 have a central role in regulating primary nitrate responses in the entire plant system.

Among the statistically significant primary nitrate–CPK target genes, CPK10, CPK30 and CPK32 modulated diverse key cellular and metabolic functions immediately activated by nitrate1,5,8,10,12,13,21. The most significantly enriched functional classes of genes were those supporting nitrate transport and assimilation; two routes of glucose–6–phosphate metabolism via the oxidative pentose-phosphate pathway and glycolysis; amino acid transport and metabolism; other transporters; carbon/nitrogen metabolism; cytokinin, auxin and abscisic acid metabolism and signalling; protein degradation; stress; signalling; and transcription (Fig. 3b, c, Extended Data Fig. 4 and Supplementary Tables 1 and 2). Notably, the universal nitrate-inducible genes responsible for the conserved nitrogen uptake and assimilation processes from Arabidopsis, rice to maize were regulated by the CPK10, CPK30 and CPK32 protein kinases1,4–5,12,13,16,28 (Extended Data Fig. 4e–g). Nearly 50 genes encoding annotation transcription factors were activated by nitrate via CPK10, CPK30 and CPK32 (Supplementary Table 2), providing potential amplification of the downstream nitrate transcriptional network1,12,13,15,20,21. Nitrate–CPK signalling repressed genes were involved in transcription, metabolism and transport (Extended Data Fig. 4b, d and Supplementary Table 2).

We carried out quantitative PCR with reverse transcription (RT–qPCR) analyses of primary nitrate–CPK target genes for eight major functional classes, including the oxidative pentose-phosphate pathway, nitrate and amino acid transporters, nitrogen metabolism, carbohydrate metabolism, signalling, cytokinin, auxin and abscisic acid metabolism, and transcription factors (Fig. 4). The gatekeeper residue in CPK10, CPK30 and human CaMKIIα (Fig. 2a), CPK32 have a central role in regulating primary nitrate responses in the entire plant system. CPK10, CPK30 and CPK32/3 are master regulators in primary nitrate signalling and integrate global gene expression to nitrate-activated metabolic and physiological responses (Figs 4, 5 and Supplementary Tables 1 and 2). Thus, CPK10, CPK30 and CPK32 are master regulators in primary nitrate signalling and integrate global gene expression to nitrate-activated metabolic and physiological responses (Figs 3, 4 and Supplementary Table 2).
KCI, NH₄⁺, Gln or KNO₃ for 5–8 days. Although NH₄⁺ or Gln could promote limited shoot greening and growth equally in wild-type and icpk seedlings, nitrate was essential for full greening and expansion of cotyledons and true leaves in wild-type seedlings. The specific icpk deficiency of cotyledon and leaf expansion in nitrate response (Fig. 4a–c) might be partially correlated with reduced expression of CYP735A2 for trans-zetin synthesis⁴⁰ (Extended Data Fig. 4f, g).

We next examined the roles of CPK10, CPK30 and CPK32 in nitrate-specific control of the root developmental program. The nitrate-specific stimulation of lateral root primordia density was reduced and lateral root elongation was severely retarded in icpk (Fig. 4c and Extended Data Fig. 6). Although ammonium supported lateral root initiation similarly in wild-type and icpk seedlings⁴³, nitrate–CPK signalling was essential for lateral root primordia progression and emergence, as well as lateral root elongation (Fig. 4c and Extended Data Fig. 6c–e). We quantified the dynamic distribution of all lateral roots from primordia stages I–VII to emergence (Em) and fully elongated (LR) stages⁴¹ in wild-type and icpk seedlings. The proportion of emerged lateral root (Em + LR) increased from 0% (4 days) to 40% (8 days) after transfer to the nitrate medium, and 3MBIP did not affect the normal development of root systems in the wild-type seedlings. By contrast, the icpk lateral root primordia were arrested most conspicuously in roots 6–8 days after transfer (Extended Data Fig. 6d–g).

Notably, the primary root length was similar between the wild-type and icpk 8-day-old seedlings on KCl or on different nitrogen media. Thus, the growth of primary roots at the early stage of development immediately after germination relied mainly on the seed nutrient reserves and sugars, with limited influence by exogenous nitrogen nutrients. The 11-day-old wild-type and icpk plants were indistinguishable on KCl, NH₄⁺ or Gln medium, displaying limited shoot and root growth and development. However, vigorous primary and lateral root growth prompted by exogenous nitrate was abolished in icpk (Figs 1c, d and 4c)¹¹. CPK10, CPK30 and CPK32 specifically defined the long-term systemic shoot developmental program and root system architecture via modulating the primary nitrate-signalling network²,¹¹.

The nitrate–CPK–NLP regulatory network

*Arabidopsis* NLP6 and NLP7 have been identified as key transcription factors of primary nitrate responses, but how they are activated by nitrate remains unknown. Analyses of transgenic plants revealed a nitrate-stimulated NLP6–MYC and NLP7–MYC mobility shift at 5–30 min, which was eliminated by phosphatase (Fig. 5a, b and Extended Data Fig. 7a, b). The nitrate-stimulated NLP7–MYC phosphorylation was greatly diminished by Gd³⁺ and La³⁺ (Fig. 5c), or by W7 (Extended Data Fig. 7c), analogous to the nitrate-responsive gene regulation (Extended Data Fig. 2a, b). Furthermore, CPK10, CPK30 and CPK32, but not the kinase-dead mutant CPK10(KM) or the subgroup I CPK11, phosphorylated NLP7 in vitro (Fig. 5d). As the Ca²⁺ chelator EGTA abolished the phosphorylation of NLP7 or histone by CPK10, CPK30 and CPK32 but not by CPK10ac, CPK30ac and CPK32ac lacking the Ca²⁺-binding domain³²–³⁵ (Fig. 5e and Extended Data Fig. 7d), CPK10, CPK30 and CPK32 were Ca²⁺ sensors and effectors to relay nitrate signalling. The nitrate–CPK–NLP6/NLP7 signalling link was further supported by the considerable overlaps between nitrate–CPK and nitrate–NLP6/NLP7 target genes as universal nitrate response marker genes.³²,³³,³⁴ (Fig. 3c, Extended Data Figs 4, 5 and Supplementary Table 2).

By aligning nine *Arabidopsis* NLPs and four orthologous *Lotus japonicus* NLPs using integrated computational and literature analyses, we identified a uniquely conserved serine (Ser205 in NLP7) as a candidate CPK phosphorylation site (Extended Data Fig. 4f, g). The serine residue is conserved across 99% of the Arabidopsis NLPS and four orthologous *Lotus japonicus* NLPS, indicating a conserved CPK phosphorylation site across species (Supplementary Table 1).
plants (Extended Data Fig. 7g). Mass spectrometry analyses confirmed that Ser205 in NLP7 was phosphorylated in vivo in the presence of nitrate (Fig. 5f). Like CPK10ac, CPK30ac and CPK32ac, NLP7 over-expression but neither kinase-dead CPK10ac(KM) nor NLP7(S205A), acted synergistically with 0.5 mM nitrate to enhance NIR–LUC activation (Fig. 1h and Extended Data Fig. 7h). Finally, nitrate-triggered NLP7 phosphorylation at Ser205 in vivo was abolished in the icpk mutant (Fig. 5g).

To validate the nitrate–CPK–NLP link further, we discovered nitrate-stimulated rapid nuclear translocation of green fluorescent protein (GFP)-tagged CPK10, CPK30 and CPK32 by confocal imaging (Fig. 6a and Extended Data Fig. 8a). NLP7–GFP but not NLP7(S205A)–GFP responded to nitrate stimulation with persistent nuclear localization in leaf cells (Fig. 6b) and in the transgenic nlp7 mutants (Extended Data Fig. 8b). Using an in vivo bimolecular fluorescence complementation (BiFC) assay, we showed that CPK10(KM) directly interacted with NLP7 in the nucleus in the presence of nitrate in protoplasts (Fig. 6c). Nitrate-stimulated nuclear relocation of NLP7–GFP was greatly diminished in icpk protoplasts (Fig. 6d). These complementary in vivo and in vitro analyses provided compelling evidence to support the signalling connections from CPK10, CPK30 and CPK32 to NLP phosphorylation in mediating primary nitrate signalling and transcription.

To identify functionally important NLP7 target genes in the nitrate–CPK–NLP signalling network, we explored genome-wide transcriptional profiling in the nitrate-responsive transient transactivation system.3,4,12,13. We discovered that NLP7–HA but not NLP7(S205A)–HA could robustly activate a wide range of putative NLP7 target genes in mesophyll cells by ectopic NLP1, NLP2, NLP5, NLP6, NLP7 and NLP9 expression12,13, as well as chromatin immunoprecipitation with DNA microarray (ChIP–chip) analyses of NLP7–GFP after 10-min nitrate treatment in transgenic seedlings3,12. Notably, we also identified potential NLP7 target genes for cell cycle initiation and auxin hormone regulators (Extended Data Fig. 9b, c). Future research will aim to determine the functions of specific NLP7 target genes in various plant organs and cell types. The identification of new NLP7 target genes was supported by the unique nlp7 phenotypes and the complementation of NLP7–GFP but not NLP7(S205A)–GFP for proliferation and growth in shoots and roots (Fig. 6e, f and Extended Data Fig. 10). The broad spectrum of nitrate-associated mutant phenotypes and primary nitrate-responsive-transcriptome defects were shared by icpk and nlp7 (refs 9, 13). As the specific serine residue is conserved among Arabidopsis and L. japonicus NLPs (Extended Data Fig. 7e), CPK10, CPK30 and CPK32 could potentially phosphorylate and activate all NLPs and possibly other transcription factors with overlapping or distinct target genes to support transcriptional, metabolic and system-wide nutrient–growth regulations differentially manifested in wild-type and conditional icpk mutant plants3,5,9,12,13,21 (Figs 2g, 3–6 and Extended Data Figs 3–10).

Discussion

We have uncovered a nitrate-coupled Ca$^{2+}$ signalling mechanism central to the plant nutrient–growth regulatory network using multifaceted approaches, encompassing an ultrasensitive Ca$^{2+}$ biosensor, a sensitized and targeted functional genomie screen, a chemical switch for conditional higher-order Arabidopsis cpk mutants, as well as integrated cellular, biochemical, genetic and systems analyses. Our findings connected a new Ca$^{2+}$–CPK–NLP signalling cascade to comprehensive nitrate responses and revealed a previously unrecognized
function of Ca<sup>2+</sup>-sensor CPKs as master regulators that orchestrate nitrate-activated signalling (Fig. 6g). As CPKs and NLPs are evolutionarily conserved from algae to land plants<sup>3,9,12,13,24–26</sup>, the nitrate-CPK-NLP signalling relay may be widespread in the plant kingdom. Our discoveries expand the biological functions of Ca<sup>2+</sup> signalling to nutrient responses essential to all life forms. Future progress is likely to identify sensors, channels and other regulators involved in generating complex Ca<sup>2+</sup> signatures in plant responses to nitrate, other nutrients, peptides, hormones and environmental cues that capitalize on continued advances of ultrasensitive Ca<sup>2+</sup> biosensors<sup>33</sup>. This work provides a molecular framework for future research on the complex interactions between nitrogen and sugar signalling pathways central to all aspects of nutrient-mediated growth regulation in plants and animals<sup>1,2,4,5,32,42–44</sup>.

**Online Content** Methods, along with any additional Extended Data display items and Online Content, can be found in the article's online version at https://doi.org/10.1038/nature24770.

**Received 21 June 2016; accepted 16 March 2017.**

**Published online 10 May 2017.**
METHODS
Plasmid constructs and transgenic lines. The 1.3-kb GCAM6 coding region was PCR amplified from the pGP-CMV-GCAM6s plasmid (Addgene)34 to generate the HBT-GCAM6-HA construct. The HBT-GCAM6-HA construct was inserted into the binary vector pCB302 (ref. 46) to generate the HBT-GCAM6-HA transgenic plants using the Agrobacterium (GV3101)-mediated floral-dip method37. Transgenic plants were selected by spraying with the herbicide BASTA. The construct expressing H5-mCherry was used as a control for protoplast co-transfection and nucleus labelling, and was obtained from J.-G. Chen38. NLS-Td-Tomato was used as a control for protoplast co-transfection and nucleus labelling, and was obtained from X. Liu. NIR-LUC was constructed as described previously31. UBQ10-GUS is a control for protoplast co-transfection and internal control; all HBT-CPK-Kac-Flag-NOS expression plasmids have been described previously32. To construct HBT-CPK-GFP-NOS, the coding regions of the CPK16, CPK30 and CPK32 cDNA were amplified and then cloned into the HBT-GFP-NOS plasmid32. HBT-CPK10(M141G)-Flag was generated by site-directed mutagenesis of the HBT-CPK10-Flag construct. To complement the cpk10 cpk30/+ mutant, a 5.5-kb DNA fragment including the promoter region (3 kb) and the coding region of CPK10 was amplified from genomic DNA, which was then cloned into the plasmid HBT-HA-NOS and mutated to generate pCPK10-CPK10(M141G)-HA-NOS. The pCPK10-CPK10(M141G)-HA-NOS construct was inserted into pCB302 and transformed into cpk10 cpk30/+ mutant plants using the Agrobacterium (GV3101)-mediated floral-dip method37. At the T1 generation, homozygous single-copy insertion lines were screened for the cpk10 cpk30 double mutant carrying pCPK10-CPK10(M141G)-HA-NOS to obtain the 3MBI-ducible icpk10,30 double mutant, which rescued the embryo lethality of the cpk10 cpk30 double mutant. The 3MBI-ducible icpk10,30,32 triple mutant expressing CPK10(M141G)-HA (designated icpk8) was generated by genetic cross to cpk32 and confirmed by molecular analyses. To construct 3SS-1-NLP6-MYC or 3SS-1-NLP7-MYC in the pCB302 binary plasmid with hygromycin B selection, the 3.6-glucononidase (GUS) gene in the 3SS-1-GUS plasmid39 was replaced with the DNA fragment encoding the full-length NLP6 or NLP7 fused to 6 copies of the MYC epitope tag in the HBT-NLPMYCHT or HBT-NLP7-MYC plasmid32. The NLP6-MYC and NLP7-MYC transgenic plants were generated by Agrobacterium (GV3101)-mediated transformation by floral dip and hygromycin B resistance selection. To construct HBT-NLPH-HA and HBT-NLP7-GFP, the 2.9 kb coding region of the NLP7 cDNA was amplified and then cloned into the HBT-NOS plasmid. HBT-NLPH(S205A)-HA and HBT-NLP7(S205A)-GFP were generated by site-directed mutagenesis. A 7.9-kb genomic DNA fragment of NLP7 was cloned into the pUC plasmid and fused with GFP at the C terminus to generate pNLP7-NLPH-GFP. The pNLP7-NLP7(S205A)-GFP construct was generated by site-directed mutagenesis. pNLP7-NLP7-GFP or pNLP7-NLP7(S205A)-GFP was then inserted into pCB302 and introduced into nlp7-1 mutant plants using the Agrobacterium (GV3101)-mediated floral-dip method37 for complementation analyses. To construct UBQ10-CPK10(KM)-YN and UBQ10-NLP7-1C, the coding regions of CPK10(KM), NLP7, YFP-N terminus and YFP-C terminus were amplified by PCR and cloned into the UBQ10-GUS plasmid. To construct pET-14-NLP7-N(1-581)-His and pET-14-NLP7(N205A)-His for protein expression, the N-terminal coding region of NLP7 and NLP7(S205A) were amplified from HBT-NLPH-HA and HBT-NLP7(S205A)-HA. All constructs were verified by sequencing. The primers used for plasmid construction and site-directed mutagenesis are listed in Supplementary Table 3.

Plant materials and growth conditions. Arabidopsis ecotype Columbia (Col-0) was used as the wild type. The cpk mutants were obtained from Arabidopsis Biological Resource Centre (ABRC)35. Homozygous T-DNA lines were identified using CPK gene-specific primers and T-DNA left-border primers. The gene-specific primers used in the Supplementary Table 3. Growth of the shoots on different exogenous nitrogen sources at different concentrations, seedlings were germinated and grown on basal medium for 4 days as described above, and then transferred to the basal medium with 0.1, 0.5, 1, 5 or 10 mM KNO3, NH4Cl, glutamine or KCl for an additional 1–7 days. For gene expression analyses with RT-qPCR and RNA-seq, 10 seedlings were germinated in one well of the 6-well tissue culture plate (Falcon) with 1 ml of the basal medium supplemented with 2.5 mM ammonium succinate as the sole nitrogen source. Plates were sealed with parafilm and placed on the shaker at 70 rpm under constant light (45 μmol m−2 s−1) at 23°C for 7 days. Before nitrate induction, seedlings were washed three times with 1 ml basal medium. Seedlings were treated in 1 ml of basal medium with KCl or KNO3 for 15 min. Seedlings were then harvested for RNA extraction with TRIzol (Thermo Fisher Scientific). To block the kinase activity of CPK10(M141G), seedlings were pre-treated with 10 μM 3MBiP in the basal medium for 2 min, and then treated with KCl or KNO3 for 15 min. For Ca2+ channel blockers and Ca2+ sensor inhibitors, assays were conducted with 2 mM CaCl2, 2 μM CdCl2, 250 μM W5 or 250 μM W7 in 1 ml of basal medium for 20 min, and then induced by 0.5 mM KCl or KNO3 for 15 min. To monitor root morphology, seedlings were germinated and grown on a basal medium supplemented with 2.5 mM ammonium succinate and 1% phytoagar under constant light (150 μmol m−2 s−1) at 23°C for 3 days. Plants were then transferred to the basal medium supplemented with 1 μM 3MBiP and 5 mM KNO3, 2.5 mM ammonium succinate, 5 mM KCl or 1 mM glutamine and grown for 5–8 days. After seedling transfer, 1 ml of 1 μM 3MBiP was added to the medium every 2 days. To monitor lateral root developmental stages, seedlings were monitored using a microscope (Leica DMS000B) with a 20× objective lens according to the protocol described previously40. To measure the primary and lateral root length, pictures were taken using a dissecting microscope (Leica MZ 16 F) with IM software and analysed by ImageJ. To compare the shoot phenotype, 8-day-old seedlings were cut above the root–shoot junction to measure the shoot fresh weight and acquire images. To analyse the cpk single-mutant phenotype, plants were germinated and grown on ammonium succinate medium for 3 days and then transferred to basal medium plates supplemented with 5 mM KNO3 for 6 days. To analyse double mutants in response to 3MBiP, plants were transferred to basal medium plates supplemented with 5 mM KNO3 and 1 μM 3MBiP for 6 days, and 3MBiP was reapplied every 2 days. Individual 9-day-old seedlings (n = 12) were collected to measure fresh weight and acquire images. To characterize the shoot phenotype of nlp7-1 and the complementation lines, around 20 seeds were germinated on the Petri dish (150 mm × 15 mm) containing 100 ml of nitrogen-free 1 × MS medium salt (Caisson), 0.1% MES, 1% sucrose, 0.7% phytoagar under constant light (150 μmol m−2 s−1) at 18°C and grown for 21 days. The shoots were collected for measurement of fresh weight and acquisition of images. For analyses of the shoot phenotype in icpk, seeds were germinated and grown on the ammonium succinate basal medium plate for 3 days and then transferred to the same medium supplemented with 1 μM 3MBiP. To analyse double mutants in response to 3MBiP, plants were transferred to basal medium plates supplemented with 5 mM KNO3 and 1 μM 3MBiP for 6 days, and 3MBiP was reapplied every 2 days. Individual 9-day-old seedlings (n = 12) were collected to measure fresh weight and acquire images. To characterize the shoot phenotype of nlp7-1 and the complementation lines, around 20 seeds were germinated on the Petri dish (150 mm × 15 mm) containing 100 ml of nitrogen-free 1 × MS medium salt (Caisson), 0.1% MES, 1% sucrose, 0.7% phytoagar and 25 mM KNO3 medium pH 5.8 under a 16 h (light)/8 h (dark) photoperiod (100 μmol m−2 s−1) at 18°C and grown for 21 days. The shoots were collected for measurement of fresh weight and acquisition of images. For analyses of the shoot phenotype in icpk, seeds were germinated and grown on the ammonium succinate basal medium plate for 3 days and then transferred to the same medium supplemented with 1 μM 3MBiP. The inhibitor 3MBiP (5 ml of 1 μM) was placed in a 15 mm Petri dish with the shoots of each mutation and grown for 21 days.

Aequorin reconstitution and bioluminescence-based quantification of Ca2+ signals in whole seedlings. Two transgenic seedlings expressing apoaequorin22 were germinated and grown in one well of a 12-well tissue culture plate (Falcon) with 0.5 ml of the basal medium supplemented with 2.5 mM ammonium succinate for 6 days. Individual plants were transferred to a luminometer cuvette filled with 100 μl of the reconstitution buffer (2 mM MES pH 5.7, 10 mM CaCl2, and 10 μM native coelenterazine from NanoLight Technology) and incubated at room temperature in the dark overnight. The emission of photons was detected every second using the luminometer BM Monolight 3010. The measurement was initiated by injection of 100 μl of 5 mM flg22 or ultrapure water into the cuvettes. Luminescence values were exported and processed using Microsoft Excel software. GCA-MP6-based Ca2+ imaging in mesophyll protoplasts and transgenic seedlings. For Ca2+ imaging in protoplasts, mesophyll protoplasts (2 × 105) in 1 ml buffer were co-transfected with 70 μg HBT-GCA-MP6 and 50 μg

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
HBT-HYS-mCherry plasmid DNA. Transfected protoplasts were incubated in 5 ml of WI buffer 4° for 4 h. Before time-lapse recording, a coverslip was placed on a 10-well chamber slide covering three-quarters of a well, and placed on the microscopy stage. Mesophyll protoplasts co-expressing GCaMP6 and HBT-mCherry (2 × 10^6 protoplasts) were spun down for 1 min at 100g. WI-Ca^2+ buffer (WI buffer plus 4 mM CaCl_2 (0.5 μl) with different stimuli (40 mM KCl, 40 mM KNO_3 or 40 mM NH_4Cl) or 80 mM CaCl_2 chelator (EGTA) were added into 1.5 μl of concentrated mesophyll protoplasts in WI buffer. The final concentration of protoplasts was estimated after centrifugation at 18,000g for 10 min at 4°C. Total proteins (20 μg) were loaded on 8% SDS–PAGE embedded with or without 0.5 mg/ml histone type III-S (Sigma) as a general CPK phosphorylation substrate. The gel was washed three times with washing buffer (25 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 5 mM NaF, 0.1 mM Na_3VO_4, 0.5 mg/ml BSA and 0.1% Triton X-100), and incubated for 20 h with three changes in the renaturation buffer (25 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 5 mM NaF and 0.1 mM Na_3VO_4) at 4°C. The gel was then incubated in the reaction buffer (25 mM Tris-HCl pH 7.5, 2 mM EDTA, 12 mM MgCl_2, 1 mM CaCl_2, 1 mM MnCl_2, 1 mM DTT and 0.1 mM Na_3VO_4) with or without 20 mM EGTA at room temperature for 30 min. The kinase reaction was performed for 1 h in the reaction buffer supplemented with 25 μM cold ATP and 50 μCi[^32]P]ATP with or without 20 mM EGTA. The reaction was stopped by extensive washes in the washing buffer (5% trichloroacetic acid and 1% sodium pyrophosphate) for 6 h. The protein kinase activity was detected on the dried gel using the Typhoon imaging system (GE Healthcare).

3MBIP synthesis. 1-Isopropyl-3-(3-methylbenzyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (3MBIP) was synthesized using the same procedures as those for a close structural analogue, 3MB-PIP (ref. 39), with comparable yields, except that iso-propylyldrazine was substituted for tert-butyldrazine.

in vitro protein kinase assays. For in vitro kinase assays with CPK10(M141G)–Flag or CPK10–Flag, 4 × 10^6 protoplasts expressing CPK10(M141G)–Flag or CPK10–Flag were lysed in 200 μl immunoprecipitation buffer that contained 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 2 mM NaF, 2 mM Na_3VO_4, 1% Triton X-100 and 1% protease inhibitor cocktail (Complete mini, Roche) and 1 mM DTT. Protein extracts were incubated with 0.5 μg anti-Flag antibody (Sigma, F1804) at 4°C for 2 h and an additional 1 h with protein G Sepharose beads (GE Healthcare). The immunoprecipitated kinase protein was washed three times with immunoprecipitation buffer and once with kinase buffer (20 mM Tris-HCl pH 7.5, 15 mM MgCl_2, 1 mM CaCl_2 and 1 mM DTT). Kinase reactions were performed for 1 h in 25 μl kinase buffer containing 1 μg histone (Sigma H5505 or H5424), 50 μM cold ATP and 2 μCi[^32]P]ATP. To block the CPK10(M141G)–Flag kinase activity, 1 μM 3MBIP or DMSO as a control was added in the 25 μl kinase buffer for 2 min before performing the kinase reaction. The reaction was stopped by adding SDS–PAGE loading buffer. After separation on a 12% SDS–PAGE gel, the protein kinase activity was detected on the dried gel using the Typhoon imaging system. For the in vitro kinase assay with CPK10(M141G)–HA isolated from icp10,30 seedlings, 12 7-day-old seedlings grown in 2 wells of a 6-well-plate with 1 ml medium (0.5 × MS, 0.5% sucrose and 0.1% MES pH 5.7) were ground in liquid nitrogen into powder and lysed in 200 μl immunoprecipitation buffer. The CPK10(M141G)–HA protein was immunoprecipitated with the anti-HA antibody (Roche, 116666006001) and protein G Sepharose beads. In vitro kinase assay with CPK10(M141G)–HA proteins was carried out as described above.

In the in vitro kinase assay with the subgroup III CPKs, Flag-tagged CPK7, CPK8, CPK10, CPK10(KM) (K92M, a kinase-dead mutation in the conserved ATP binding domain), CPK13, CPK30 and CPK32 were transfection into mesophyll protoplasts and purified with 1 μg anti-Flag antibody conjugated to protein G Sepharose beads as described above. CPK11–Flag from subgroup I was used as a negative control to demonstrate the specificity of NLPl^7 as a substrate for only subgroup III CPKs. NLPl^7–His (~1 μg) purified from Escherichia coli or histone type III-S (2 μg) was used as substrate in the in vitro kinase assay. Kinase reactions were performed for 1 h at 28°C in 25 μl kinase buffer containing 5 μM cold ATP and 60 μCi[^32]P]ATP, which greatly enhanced the CPK activity. To reduce the background caused by free[^32]P]ATP in the gel, 50 μM cold ATP was added to the kinase reaction before sample loading in 10% NLPl^7–His or 12% (His) SDS–PAGE gel. To demonstrate that the kinetic activities of CPK10, CPK30 and CPK32 were Ca^2+-dependent, 4 × 10^6 (CPK10 or CPK10ac) or 10^6 (CPK30, CPK32, CPK32ac or CPK32ac) protoplasts expressing CPKs for 12 h instead of 6 h (to increase the yield of CPK proteins) were lysed in 200 μl (CPK10) or 400 μl (CPK30 or CPK32) of immunoprecipitation buffer. The CPK proteins were immunoprecipitated with the AKTA FPLC system. Purified proteins were buffer exchanged into PBS using PD-10 Desalting Columns (GE Healthcare), and then concentrated by Amicon Ultra-4 Centrifugal Filter Unit with Ultraloc-10 membrane (EMD Millipore).

In-gel protein kinase assay. Around 10^6 protoplasts were incubated in WI buffer (5 μl) in Petri dishes (9 × 9 cm) for 4 h before induction with 10 mM KCl or KNO_3 for 10 min. Protoplasts were harvested and lysed in 200 μl of extraction buffer: 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 1% Triton X-100, 1× protease inhibitor cocktail (Complete mini, Roche) and 1 mM DTT. The protein extract supernatant was obtained after centrifugation at 18,000g for 10 min at 4°C. Total proteins (20 μg) were loaded on 8% SDS–PAGE embedded with or without 0.5 mg/ml histone type III-S (Sigma) as a general CPK phosphorylation substrate. The gel was washed three times with washing buffer (25 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 5 mM NaF, 0.1 mM Na_3VO_4, 0.5 mg/ml BSA and 0.1% Triton X-100) and incubated for 20 h with three changes in the renaturation buffer (25 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 5 mM NaF and 0.1 mM Na_3VO_4) at 4°C. The gel was then incubated in the reaction buffer (25 mM Tris-HCl pH 7.5, 2 mM EDTA, 12 mM MgCl_2, 1 mM CaCl_2, 1 mM MnCl_2, 1 mM DTT and 0.1 mM Na_3VO_4) with or without 20 mM EGTA at room temperature for 30 min. The kinase reaction was performed for 1 h in the reaction buffer supplemented with 25 μM cold ATP and 50 μCi[^32]P]ATP with or without 20 mM EGTA. The reaction was stopped by extensive washes in the washing buffer (5% trichloroacetic acid and 1% sodium pyrophosphate) for 6 h. The protein kinase activity was detected on the dried gel using the Typhoon imaging system (GE Healthcare).

The kinetic activities of CPK10, CPK30 and CPK32 were determined with the region of interest (ROI) function for each protoplast. The intensity data were exported and processed using Microsoft Excel software. The images were exported and processed using Adobe Photoshop software. To make a video, individual images were cropped using Adobe Photoshop software and saved in JPEG format. The videos were generated using ImageJ with the cropped images. For Ca^2+ imaging with the GCaMP6 transgenic seedling cotyledons, 5 seedlings were germinated in 1 well of a 6-well tissue culture plate (Falcon) with 1 ml of the basal medium supplemented with 2.5 mM ammonium succinate for 7 days. A chamber was made on microscope slides between two strips of the invisible tape (0.5 cm × 3 cm) and filled with 150 μl of the basal medium. A cotyledon of the 7-day-old seedling was cut in half using a razor blade and embedded in the medium. A thin layer of cotton was placed on top of the cotyledon to prevent moving. The coverslip was placed on the sample and fixed by another two strips of the invisible tape. The cotyledon was allowed to recover on the slide for 10 min. Confocal imaging was acquired using the Leica laser scanning confocal system (Leica TCS NT confocal microscope, SP1). The mesophyll cells in the cotyledon were targeted for Ca^2+ imaging at the focal point. Basal medium (200 μl) with 10 mM KCl, 10 mM KNO_3 or 20 mM EGTA was loaded along one edge of the slide and imaged via the Leica AF software on a Leica DM5000B microscope with the 20× objective lens. The exposure time for GCaMP6 was set at 1 s and recorded every 2 s to generate 199 frames. The exposure time was set at 45 ms for the bright field and 1 s for the mCherry signal. The fluorescence intensity was determined with the region of interest (ROI) function for each protoplast. The intensity data were exported and processed using Microsoft Excel software. The images were exported and processed using Adobe Photoshop software.
anti-Flag antibody (0.5 μg for CPK10 or CPK10ac, and 2 μg for CPK30, CPK32ac or CPK32ac) conjugated to protein G Sepharose beads. The immunoprecipitated CPKs were washed three times with immunoprecipitation buffer and twice with EGTA kinase buffer (20 mM Tris-HCl pH 7.5, 15 mM MgCl2, 15 mM EGTA and 1 mM DTT). Kinase reactions were performed for 1 h at 28 °C in 25 μl kinase buffer or EGTA kinase buffer containing 5 μM cold ATP and 6 μCi [γ-32P]ATP and purified NLP7–HIS (~1 μg), NLP7–N (1–581 amino acids) (~0.8 μg), NLP7–N(S205A) (~0.8 μg), or histone type III-S (2 μg). After performing the kinase enrichment reaction, 50 μl cold ATP was added to reduce the background caused by free [γ-32P]ATP. The reaction was stopped by adding SDS–PAGE loading buffer after separation on a 12% SDS–PAGE gel (histone type III-S) or 10% (NLP7–HIS or NLP7–N–HIS) SDS–PAGE gel, the protein kinase activity was detected on the dried gel using the Typhoon imaging system. Substrate was stained with InstantBlue Protein Stain (C.B.S. Scientific). The expression levels of CPK or CPKac proteins were monitored by immunoblot with anti-Flag-HRP (Sigma, A8592; 1:4,000) antibody. CPKac proteins without the Ca2⁺-binding EF-hand domains provided constitutive kinase activities that were insensitive to EGTA. The sensitivity of CPK10, CPK30 and CPK32 to EGTA in kinase assays demonstrated their functions as Ca2⁺ sensors in nitrate signalling, which was further supported by the lack of NLP7–HA phosphorylation and the nuclear retention of NLP7–GFP in icpk mutant cells. Importantly, NLP7(S205A) lost nitrate-induced phosphorylation, nuclear localization, NLR-LUC activation, and endogenous target gene activation in wild-type protoplasts and seedlings.

RNA isolation, RT–PCR and RT–qPCR. RNA isolation, RT–PCR and RT–qPCR were carried out as described previously11. The primers used for RT–PCR and RT–qPCR are listed in Supplementary Table 5. TUB4 was used as a control in wild-type and icpk mutants. The relative gene expression was normalized to the expression of UBIQ10. Triplicate biological samples were analysed with consistent results.

RNA-seq analyses. We chose the early time point to minimize secondary target genes and the complexity that negative feedback would have introduced, including indirect effects from assimilation of nitrate and the subsequent activation of transcriptional repressors1,3,4,8,10,13. Seven-day-old wild-type and icpk seedlings were pretreated with 10 μM 3MBiP for 2 min and then treated for 15 min with either 10 mM KCl or 10 mM KNO3. Total RNA (0.5 μg) was used for preparing the library with the Illumina TruSeq RNA sample Prep Kit v2 according to the manufacturer’s guidelines with 9 different barcodes (triplicate biological samples). The libraries were sequenced for 50 cycles on an Illumina HiSeq 2500 rapid mode using two lanes of a flow cell. The sequencing was performed at MGHi Next Generation Sequencing Core facility (Boston, USA). Fastq files, downloaded from the core facility, were used for data analysis. The quality of each sequencing library was assessed by examining fastq files with FastQC. Reads in the fastq file were first aligned to the Arabidopsis genome, TAIR10, using TopHat2. HTSeq2 was used to determine the reads per gene. Finally, DESeq2 (ref. 53) analysis was performed to determine differential expression44. For HTSeq-normalized counts in each sample, differentially expressed genes were determined for wild-type KNO3 versus wild-type KIC and icpk KNO3 versus wild-type KNO3. The differential expression analysis in DESeq2 uses a generalized linear model of the form

\[ \log(q_i) = \kappa_0 + \kappa_1 \times \mu_i \]

For the differentially expressed genes, this equation was used to calculate fold changes in the relative expression level, which was then used to identify enriched gene functions using the Enrichr tool56. The gene ontology and biological process pathways were used as the input for the Enrichr tool. The list of enriched gene functions, we used the Classification SuperViewer Tool on the BAR website (http://bar.utoronto.ca/toools/cgi-bin/toools_classification_superviewer.cgi) with the MapMan classification source option. Analyses of enriched functional categories with nitrate upregulated and downregulated genes were performed using the MapMan classification source option on the Classification SuperViewer Tool with manual annotation based on literature. The fold enrichment is calculated as follows: (number in clasSupat_set/number of totalSupat_set) (number in clasReference_set/number of totalReference_set). The P value is calculated in Excel using a hypergeometric distribution test. The data in Extended Data Fig. 4c and d were sorted by fold enrichment with a P < 0.05 cut-off. For the biological duplicate RNA-seq experiments for identifying NLP7 target genes in the mesophyll protoplast transient expression system, 500 μg HBT-NLP7–HA, HBT-NLP7(S205A)–HA or control plasmid DNA was transfected into 106 protoplasts and incubated for 4.5 h. Total RNA (0.5 μg) was used to construct the libraries with six different barcodes (biological duplicate samples) as described above. The library names and contents are listed in Supplementary Table 1 and described above. Differentially expressed genes were determined with DESeq2 on NLP7 versus Ctrl (Control) and NLP7(S205A) versus Ctrl. Results were imported into Microsoft Excel for filtering (log2 ≥ 1 cut-off) and generating heatmaps.

Immunoblot analyses of nitrate-induced phosphorylation of NLP6 and NLP7 in vivo. Transgenic seedlings expressing NLP6–MYC or NLP7–MYC were germinated and grown in basal medium containing 0.5 mM ammonium succinate as a sole nitrogen source (0.01% MES–KOH, pH 5.7) for 4 days at 23 °C under continuous light (60 μmol m⁻² s⁻¹). After replacement with fresh medium supplemented with 10 mM KCl or KNO3, the seedlings were collected after incubation for 5, 10 or 30 min. To examine the effects of Ca2⁺ channel blockers and Ca2⁺ sensors inhibitors, the 4-day-old seedlings were placed in fresh basal medium supplemented with 2 mM LaCl3, 2 mM CdCl2, 250 μM W5 or 250 μM W7 for 20 min and induced by 10 mM KCl or KNO3. The seedlings were washed, frozen in liquid nitrogen and ground using a Multibeads Shocker (Yasui Kikai). The ground samples were suspended in 20 volume of 1× Laemmli sample buffer supplemented with 1× EDTA-free protease inhibitor cocktail (Roche) and heated at 95 °C for 30 s. Samples were then spun down and the supernatant was subjected to SDS–PAGE and immunoblottting with anti-MYC (Millipore, 05-419; 1:1,000) and anti-histidine H (Abcam, ab1791; 1:5,000) antibodies. For calf intestinal alkaline phosphatase (CIP) treatment, proteins in 1.2-fold CIP buffer (60 mM Tris-HCl pH 8.0, 120 mM NaCl, 12 mM MgCl2, 1.2 mM DTT, 2.4-fold concentration of EDTA-free Protease Inhibitor Cocktail) were mixed with CIP solution (New England Biolabs, M0290, 10 μl μl⁻¹) at a ratio of 5 (CIP buffer):1 (CIP solution) and incubated at 37 °C for 30 min. Heat-inactivated CIP was mixed as a control treatment. The reactions were stopped by adding an equal volume of 2× Laemmli sample buffer and heating at 95 °C for 30 s. To demonstrate that nitrate-induced NLP7 phosphorylation was abolished in icpk by protein mobility shift in SDS–PAGE, 4× 105 protoplasts isolated from wild-type or icpk seedlings were transfected with 20 μg NLP7–HA or NLP7(S205A)–HA. To block CPK10 (M141G) activity in icpk, 10 μg 3MBiP was added in the incubation buffer (W1) after transfection. After expressing protein for 4.5 h, protoplasts were induced by 10 mM KCl or KNO3 for 15 min. Protoplasts were spun down and re-suspended in 40 μl 1× Laemmli sample buffer. Samples (10 μl) were separated in a 6% SDS–PAGE resolving gel without a stacking gel layer. After transferring proteins to the PVDF membrane, the NLP7 (wild-type and S205A) proteins were detected with an anti-ha-peroxidase (Roche, 11667475001; 1:2,000). Rubisco was detected by an anti-rubisco antibody (Sigma, GW23153; 1:5,000) as a loading control.

Detection of Ser205 phosphorylation in NLP7 in vivo. Transformation of T87 cell suspension culture derived from a seedling of A. thaliana (Heynh.) ecotype WI was conducted as the SSU5-NLP7–MYC construct in the pCB302 binary plasmid carrying the hygromycin B selection marker gene. Transformants mediated by Agrobacterium (GY31001) were selected on agar plates (IPL medium, 3 g l⁻¹ gellan gum, 500 μg l⁻¹ carbendicillin and 20 mg l⁻¹ hygromycin), and the transformants were maintained in liquid IPL medium as described previously37. T87 cells expressing NLP7–MYC were incubated in nitrogen-free IPL liquid medium for 2 days, and then 10 mM KNO3 was added into the medium. After 30 min treatment, the T87 cells (approximately 4 g frozen weight) were frozen in liquid nitrogen and homogenized with Multi-beads Shocker (Yasui Kikai) in 10 ml sodium phosphate (pH 7.5) buffer containing 50 mM NaCl, 10% glycerol, 1× Complete Protease Inhibitor Cocktail and 1× PhosSTOP (Roche). Cell lysates obtained were incubated with anti-MYC antibodies crosslinked to Dynabeads (Invitrogen). Trapped proteins were eluted by 1× Laemmli sample buffer and separated by SDS–PAGE. Gel pieces containing NLP7–MYC were recovered and subjected to in-gel double digestion with trypsin (10 ng μl⁻¹) and chymotrypsin (10 ng μl⁻¹) (Promega). NanoLC–ESI–MS/MS analysis was performed as described previously37 with minor modifications.

NLP7, CPK10, CPK30 and CPK32 nuclear localization analyses. To analyse NLP7 nuclear retention triggered by nitrate in protoplasts, nitrate-free mesophyll protoplasts (4× 105 protoplasts in 200 μl) were co-transfected with 20 μg NLP7–GFP or NLP7(S205A)–GFP and 10 μg HBT-HY5-MCherry plasmid DNA and incubated for 6 h. Mesophyll protoplasts were spun down for 1 min at 100,000 g for 20 min and washed with 10 mM KCl or KNO3, added into mesophyll protoplasts for 30 min. The treated protoplasts were loaded onto slides and imaged with the 20× objective lens on a Leica DM5000B microscope operated with the Leica AF software.
The images were collected and processed using Adobe Photoshop software. To analyse NLP7-GFP nuclear retention triggered by nitrate in transgenic lines, NLP7–GFP/nlp7-1 and NLP7(S205A)–GFP/nlp7-1 seedlings were germinated and grown on the basal medium supplemented with 2.5 mM ammonium succinate and with 1% phytosugar under constant light (150 μmol m⁻² s⁻¹) at 23 °C for 5 days. Plants were placed on the slide as described above and stimulated by 10 mM KNO₃. Confocal images were acquired as described for GCaMP6-based Ca²⁺ imaging in transgenic seedlings. To analyse CPK10, CPK30 and CPK32 nuclear localization in response to nitrate, nitrate–free mesophyll protoplasts (4 × 10⁴ protoplasts in 200 μl) were co-transfected with 20 μg CPK10–GFP, CPK30–GFP or CPK32–GFP and 10 μg HBT-HYS-mCherry plasmid DNA and incubated for 12 h. Protoplasts were then treated with 10 mM KNO₃ for 5 min. Confocal imaging was acquired using the Leica Application Suite X software on a Leica TCS SP8 (Leica) confocal microscope with the 40× objective lens. To obtain fluorescence images, the excitation was set to 489 nm (GFP) and 587 nm (mCherry), and images at emissions 508 nm (GFP) and 610 nm (mCherry) were collected. The scanning resolution was set to 1.024 × 1.024 pixels. The images were collected and processed using Adobe Photoshop software. To analyse NLP7–GFP nuclear retention in wild-type and icpk seedlings, nitrate–free mesophyll protoplasts (4 × 10⁴ protoplasts in 200 μl) were co-transfected with 20 μg NLP7–GFP and 4 μg HBT-Td-Tomato plasmid DNA and incubated for 12–16 h. The transfected protoplasts were treated with inhibitor 10 μM 3MBiP 30 min before nitrate induction. Protoplasts were treated with 10 mM KNO₃ for 15 min in the presence of 10 μM 3MBiP of W1 buffer. The images were acquired as described above for the NLP7 nuclear retention in protoplasts.

**BiFC assay.** Nitrate-free mesophyll protoplasts (4 × 10⁴ protoplasts in 200 μl) were co-transfected with 18 μg UBQ10–CPK10(KM)-YN, UBQ10-NLP7-YC, and 4 μg HBT-HYS-mCherry plasmid DNA, and incubated for 12–18 h. Protoplasts were then treated with 10 mM KNO₃ for 2 h. Confocal images were acquired as described above for CPK localization in response to nitrate.

**Statistical analysis.** The chosen sample sizes for all experiments were empirically determined by measuring the mean and s.d. for the sample population in pilot experiments, and then calculated (the 1-sample Z-test method, two-sided test) with the aim to obtain the expected mean of less than 25% significant difference with the alpha value ≤ 0.05 and the power of the test ≥ 0.80. For multiple comparisons, data were first subjected to one-way or two-way ANOVA, followed by Tukey’s multiple comparisons test to determine statistical significance. To compare two groups, a Student’s t-test was used instead. To compare wild-type and icpk lateral root development, data were categorized into two groups, and then subjected to a chi-square test, as indicated in the figure legends. Experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment.

**Data availability.** RNA-seq data are available at the Gene Expression Omnibus (GEO) under accession number GSE73437. The Source Data for blots, gels and histograms are provided in the Supplementary Information. All other data are available from the corresponding author upon reasonable request.


Nitrate promotes plant development and induces Ca\(^{2+}\) signatures in leaves and roots. **a**, Nitrate promotes shoot and root development. Plants were germinated without an exogenous nitrogen source for 4 days and then transferred to the plates supplemented with different concentrations of KNO\(_3\), NH\(_4\)Cl or glutamine for 7 days. Scale bars, 1 cm. The experiments were repeated twice with 10 seedlings for each treatment with consistent results. **b, c**, Distinct Ca\(^{2+}\) signatures induced by nitrate and flg22 in aequorin transgenic plants. Arabidopsis transgenic seedlings constitutively expressing the Ca\(^{2+}\) reporter protein apoaequorin were grown in liquid medium containing 2.5 mM ammonium succinate as the sole nitrogen source for 6 days. Aequorin was reconstituted with 10 \(\mu\)M coelenterazine overnight in the dark. The results are presented as relative light units (RLU) in response to 10 mM KCl or KNO\(_3\) (b) or to 100 nM flg22 or water (c) at intervals of 1 s. Error bars, ± s.e.m., \(n = 10\) seedlings. The experiments were repeated three times with similar results. The RLU value is cut-off at 3,500. **d**, NRT1.1 is highly expressed in shoots and mesophyll protoplasts. The signal counts of the genes in roots and shoots were derived from previously published microarray data\(^5\). The signal counts of the genes for mesophyll protoplasts were derived from previously published microarray data\(^6\). LHC\(_{B2.2}\) serves as a leaf-specific expression control. The control gene UBQ10 is constitutively and highly expressed in roots, shoots and mesophyll protoplasts. Error bars, s.d., \(n = 3\) biological replicates from mesophyll protoplasts. **e**, Nitrate induction of the endogenous NIR gene as a primary nitrate-responsive marker gene in seedlings and mesophyll protoplasts. NIR expression was quantified by RT–qPCR analysis. Arabidopsis seedlings or mesophyll protoplasts were treated with 10 mM KCl or KNO\(_3\) for 2 h. Error bars, s.d., \(n = 3\) biological replicates. **f, g**, Time-lapse images of nitrate-stimulated Ca\(^{2+}\) signalling in roots of intact transgenic GCaMP6 plants. The entire time-lapse recording of Ca\(^{2+}\) signals stimulated by 10 mM KCl or KNO\(_3\) in the root tip (f) or the elongated region (g) is shown in Supplementary Videos 3 and 4. Seedlings were grown on basal medium without nitrogen for 4 days and then stimulated by KCl or KNO\(_3\). Scale bars, 10 \(\mu\)m. The experiments were repeated three times with 10 seedlings for each treatment with consistent results. Source Data for **d** and **e** can be found in the Supplementary Information.
Extended Data Figure 2 | See next page for caption.
Extended Data Figure 2 | Calcium mediates the nitrate response in seedlings and mesophyll protoplasts. a, Ca^{2+} channel blockers diminish primary nitrate-responsive transcription. RT–qPCR analyses with 7-day-old seedlings. 0.5 mM KNO3, 15 min. Error bars, s.d., n = 3 biological replicates. *P < 0.05, **P < 0.0001 (two-way ANOVA with Tukey’s multiple comparisons test). b, An antagonist of Ca^{2+} sensors (W7) inhibits primary nitrate-responsive transcription. Error bars, s.d., n = 3 biological replicates. *P < 0.05, **P < 0.0001 (two-way ANOVA with Tukey’s multiple comparisons test). c, Nitrate stimulates putative endogenous CPKs in an in-gel kinase assay. 10 mM KNO3, 10 min. d, Time-course analysis of NIR-LUC activity in response to nitrate induction. Mesophyll protoplasts co-transfected with NIR-LUC and UBQ10-GUS (as the internal control) were incubated in WI buffer for 4 h and then induced by 0.5 mM KCl or KNO3 for 0.5, 1, 2 and 3 h. The fold change is calculated relative to the value of KCl treatment at each time point. Error bars, s.d., n = 3 biological replicates. e, Nitrate-specific induction of NIR-LUC expression. Transfected mesophyll protoplasts were incubated in WI buffer for 4 h and then induced by 0.5 mM KCl or different nitrogen sources for 2 h. Error bars, s.d., n = 3 biological replicates. f, Sensitive regulation of NIR-LUC by nitrate. Transfected mesophyll protoplasts were incubated in WI buffer for 4 h and then induced by different concentration of KCl or KNO3 for 2 h. Error bars, s.d., n = 3 biological replicates. g, Relation tree of Arabidopsis CPK proteins. The relation tree was generated by ClustalX and Treeview algorithms using the complete protein sequences of CPKs. The subgroup III CPKs that enhanced NIR-LUC activity by more than two-fold are highlighted. Genes encoding CPK14 and CPK24 are not expressed in mesophyll cells. Source Data for a, b, d–f can be found in the Supplementary Information.
Extended Data Figure 3 | See next page for caption.
Extended Data Figure 3 | Analyses of single and double cpk and icpk mutants in subgroup III CPKs. a, b, The cpk T-DNA insertion lines. All cpk mutants were isolated and confirmed by PCR analysis of genomic DNA using gene-specific primers and a T-DNA left-border primer. Lines represent introns or promoters, whereas dark and light grey boxes represent exons and untranslated regions, respectively. Arrows represent primers used for genotyping (see Supplementary Table 4). c, RT–PCR analysis of CPK transcripts in cpk mutants. TUB4 is the housekeeping control gene. d, Analyses of nitrate-responsive marker gene expression in cpk mutants. Seedlings (7-day-old) were induced by 0.5 mM KCl or KNO₃ for 15 min. Relative expression of nitrate-responsive marker genes was analysed by RT–qPCR and normalized to the expression of UBQ10. The expression level is calculated relative to the value of wild-type seedlings treated with KCl. Error bars, s.d., n = 3 biological replicates. e, Single and double cpk mutants lack an overt phenotype. Plants were germinated and grown on the ammonium succinate medium for 3 days and then transferred to basal medium plates supplemented with 5 mM KNO₃ for 6 days. To analyse the chemical analogue-sensitive mutants, wild-type and icpk10,30 seedlings were transferred to basal medium plates supplemented with 5 mM KNO₃ and 1 µM 3MBiP for 6 days, and 3MBiP was reapplied every 2 days after transfer. Scale bar, 1 cm. Images are representative of 10 seedlings. f, g, The average fresh weight of 9-day-old single and double cpk mutants. Error bars, s.d., n = 12 seedlings. h, The average fresh weight of 9-day-old double cpk mutants and icpk supplemented with or without 3MBiP. Error bars, s.d., n = 12 seedlings. i, j, Primary nitrate-responsive gene expression is reduced in cpk double mutants. RT–qPCR analyses with 7-day-old seedlings. 0.5 mM KNO₃, 15 min. Error bars, s.d., n = 3 seedlings. *P < 0.05, **P < 0.0001 (two-way ANOVA with Tukey’s multiple comparisons test). Source Data for d, i and j can be found in the Supplementary Information.
Extended Data Figure 4 | RNA-seq and qRT–PCR data analyses and functional classification. Biological triplicate RNA-seq experiments were performed and analysed with DESeq2. a, Nitrate–CPK-downregulated genes. Dark grey, nitrate–CPK target genes ($q \leq 0.05$). b, Classification of nitrate–CPK-downregulated genes. The MapMan functional categories for nitrate-downregulated genes are presented. c, Enriched functional categories of nitrate-upregulated genes. The fold enrichment is calculated as follows: (number of classified_input_set/number of total_input_set)/(number of classified_reference_set/number of total_reference_set). The P value is calculated in Excel using a hypergeometric distribution test. The categories were sorted by fold enrichment with a P < 0.05 cut-off. e, Nitrate–CPK target genes regulate nitrogen transport and metabolism. f, RT–qPCR analyses of nitrate–CPK target genes in eight functional classes in seedlings. 10 mM KNO$_3$, 15 min. Error bars, s.d., n = 3 biological replicates. NIA1/2, NIR, NRT2.1/2.2, G6PD2/3, GLN, GDH3, UPM1, FD3 and FNR1/2 genes were regulated by the CPK10, CPK30 and CPK32 protein kinases. g, The fold changes of expression levels of nitrate-upregulated genes in wild-type and icpk seedlings listed in f. The table provided the Source Data for the histograms presented in f. n = 3 biological replicates.
Extended Data Figure 5 | Primary nitrate-upregulated genes are present in diverse experimental systems. Venn diagrams (http://www.cmbi.ru.nl/cdd/biovenn/) were used to present the comparison and overlaps between the list of primary nitrate-upregulated genes defined in this study and the nitrate-upregulated genes at 20 min defined by previously published gene sets. Red, 394 nitrate-upregulated genes identified in this study with a log$_2$ ≥ 1 and q ≤ 0.05 cut-off; light red, 992 nitrate-upregulated genes in this study with a q ≤ 0.05 cut-off; dark blue, 338 nitrate-upregulated genes from ref. 4 with a log$_2$ ≥ 1 cut-off for both biological duplicate datasets; green, 366 nitrate-upregulated genes from the ref. 10 dataset with a log$_2$ ≥ 1 cut-off; light blue, 227 nitrate-upregulated genes from ref. 13 with a log$_2$ ≥ 1 cut-off. Gene numbers in each group and the percentage of overlapped nitrate-upregulated genes in previously published datasets are shown.
Extended Data Figure 6 | See next page for caption.
Extended Data Figure 6 | Quantitative analyses of root growth phenotype in wild-type and icpk seedlings in response to ammonium and nitrate. a, The icpk mutant displays defects in nitrate-stimulated lateral root establishment. Wild-type and icpk mutant seedlings were germinated and grown on ammonium succinate medium for 3 days, and then transferred to a plate supplemented with 5 mM KNO₃, 2.5 mM ammonium succinate, 5 mM KCl or 5 mM glutamine in the presence of 1 μM 3MBiP for 5 days. Scale bars, 1 cm. Images are representative of 6 seedlings. b, Primary root (PR) length was similar in 8-day-old wild-type and icpk seedlings. Error bars, s.d., n = 16 seedlings. c, Lateral root primordium (LRP) density decreased significantly in 8-day-old icpk seedlings in response to nitrate. Error bars, s.d., n = 15 seedlings. *P < 0.05 (Student's t-test). d, Lateral root (LR) length was markedly reduced in icpk seedlings in the presence of nitrate. Error bars, s.d., n = 10 seedlings. *P < 0.05 (Student's t-test). e, The development of lateral roots is severely retarded in icpk. The developmental stages of the third lateral root in 6-day-old wild-type and icpk seedlings induced by nitrate for 3 days are shown. Scale bars, 100 μm. Images are representative of 6 seedlings. f, Time-course analyses of icpk defects in nitrate-specific lateral root development stages I–VII. Em, emerged primordia. Error bars, s.e.m., n = 16 seedlings. g, Chi-square test of wild-type and icpk lateral root development. Wild-type and icpk seedlings were compared on two categories, early lateral root development stages before emergence (stage I–VII) and afterwards (Em + LR). The low P value indicates the high level of association between the genotype and development stages.
Extended Data Figure 7 | Nitrate-induced NLP phosphorylation.

**a**, Nitrate-induced mobility shift of MYC-tagged NLP6. Transgenic seedlings expressing MYC-tagged NLP6 were grown in liquid medium containing 0.5 mM ammonium succinate as a sole nitrogen source for 4 days and then treated with 10 mM KCl or KNO₃ for indicated periods. Immunoblot analysis was carried out with proteins extracted from the seedlings using anti-MYC and anti-histone H3 (HIS) antibodies. **b**, Effect of alkaline phosphatase treatment on mobility shift of MYC-tagged NLP6. Proteins from seedlings treated with 10 mM KNO₃ for 0 or 30 min were subjected to CIP treatment. The experiments were repeated twice with consistent results. **c**, An antagonist of Ca²⁺ sensors (W7) diminished nitrate-triggered phosphorylation of NLP7. **d**, Phosphorylation of histone by CPK10, CPK30 and CPK32 is Ca²⁺-dependent. **e**, Alignment of the amino acid sequences around the conserved CPK phosphorylation site in all NLPs from *A. thaliana* and *L. japonicus* (Lj). Conserved amino acid residues are indicated by black boxes. The CPK phosphorylation motif indicated by an underline was identified by multiple web-based bioinformatics tools and literature analysis with a candidate serine (Ser205 in NLP7) that is uniquely conserved in nine *Arabidopsis* NLPs and four orthologous *L. japonicus* NLPs (outlined in red), but not in *L. japonicus* NIN. LjNIN, a variant of NLP, which evolved specifically for symbiotic nitrogen fixation in legumes, lacks a CPK phosphorylation site. **f**, Ser205 in NLP7 is the phosphorylation site for CPK10, CPK30 and CPK32. **g**, Nitrate-induced mobility shift was abolished for NLP7(S205A). **h**, Overexpression of NLP7 and CPK10ac showed similar synergism with nitrate for NIR-LUC activation in protoplast transient assays. NLP7 or CPK10ac alone was not effective to enhance NIR-LUC expression without nitrate. CPK10(KM) lacking kinase activity and NLP7(S205A) lacking the CPK10, CPK30 and CPK32 phosphorylation site served as negative controls. NLP7 or CPK10ac protein expression was detected by immunoblot analyses before dividing protoplasts equally, and treated with 0.5 mM KCl or KNO₃ for 2 h. UBQ10-GUS is a transient expression control. Error bars, s.d., n = 5 biological replicates.
Extended Data Figure 8 | The CPK phosphorylation residue Ser205 is required for NLP7 nuclear retention triggered by nitrate at the plant root tip. a, CPK–GFPs are not processed in response to nitrate. Proteins from CPK–GFP-transfected protoplasts were analysed by immunoblots with an anti-GFP antibody. b, Confocal image of NLP7–GFP or NLP7(S205A)–GFP in transgenic nlp7-1 complementation plants in response to nitrate. GFP images recorded at 0 or 8 min after 10 mM KNO₃ induction are shown. Scale bars, 100 μm. The experiments were repeated 3 times with 10 seedlings for each line with consistent results.
Extended Data Figure 9 | Nitrate enhancement of proliferation in the lateral root primordia. a, b, Ser205 is crucial for NLP7-mediated transcriptional activation of target genes with diverse functions. Genome-wide transcriptional profiling by RNA-seq was performed with mesophyll protoplasts expressing NLP7–HA or NLP7(S205A)–HA or the control plasmid for 4.5 h. Red, NLP7 target genes identified by both ChIP–chip and DNA affinity purification sequencing (DAP-seq); black, ChIP–chip only; grey, DAP-seq only. c, Normalized HTSeq read counts of NLP7-activated genes (listed in a and b) from RNA-seq experiments. Normalized read counts of NLP7-activated genes calculated as the original HTseq counts divided by the normalization factors were extracted after DESeq2 analysis.
Extended Data Figure 10 | Complementation analyses of nlp7-1 with NLP7–GFP or NLP7(S205A)–GFP in transgenic Arabidopsis plants.

a. The shoot fresh weight of wild-type, nlp7-1, NLP7-GFP/nlp7-1 and NLP7(S205A)–GFP/nlp7-1 shoots. Error bars, s.e.m., n = 10 seedlings. **P ≤ 0.0001 versus wild-type control (one-way ANOVA with Tukey’s multiple comparisons test). Plants were grown on 25 mM KNO₃ medium for 21 days. Data from three independent complement lines are presented.

b. The root fresh weight of 11-day-old wild-type, nlp7-1, NLP7–GFP/nlp7-1 and NLP7(S205A)–GFP/nlp7-1. Seedlings were germinated on the ammonium succinate medium for 3 days and then transferred to the plates supplemented with 5 mM KNO₃ for 8 days. Error bars, s.e.m., n = 10 seedlings. **P ≤ 0.0001 versus wild-type control (one-way ANOVA with Tukey’s multiple comparisons test).
Reproduced with permission of copyright owner. Further reproduction prohibited without permission.