Feedback inhibition of AMT1 NH$_4^+$-transporters mediated by CIPK15 kinase

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Abstract

**Background:** Ammonium (NH$_4^+$), a key nitrogen form, becomes toxic when it accumulates to high levels. Ammonium transporters (AMTs) are the key transporters responsible for NH$_4^+$ uptake. AMT activity is under allosteric feedback control, mediated by phosphorylation of a threonine in the cytosolic C-terminus (CCT). However, the kinases responsible for the NH$_4^+$-triggered phosphorylation remain unknown.

**Results:** In this study, a functional screen identified protein kinase CBL-Interacting Protein Kinase15 (CIPK15) as a negative regulator of AMT1;1 activity. CIPK15 was able to interact with several AMT1 paralogs at the plasma membrane. Analysis of AmTryoshka, an NH$_4^+$ transporter activity sensor for AMT1;3 in yeast, and a two-electrode-voltage-clamp (TEVC) of AMT1;1 in Xenopus oocytes showed that CIPK15 inhibits AMT activity. CIPK15 transcript levels increased when seedlings were exposed to elevated NH$_4^+$ levels. Notably, cipk15 knockout mutants showed higher $^{15}$NH$_4^+$ uptake and accumulated higher amounts of NH$_4^+$ compared to the wild-type. Consistently, cipk15 was hypersensitive to both NH$_4^+$ and methylammonium (MeA) but not nitrate (NO$_3^-$).

**Conclusion:** Taken together, our data indicate that feedback inhibition of AMT1 activity is mediated by the protein kinase CIPK15 via phosphorylation of residues in the CCT to reduce NH$_4^+$-accumulation.

**Keywords:** Arabidopsis thaliana, Ammonium, Protein kinase, Phosphorylation, Transporter

**Background**

As a key building block of nucleic acids, amino acids, and proteins, nitrogen is an essential nutrient. In plants, nitrogen supply can limit or inhibit growth, development, and crop yield when below or above the optimal range. Ammonium (NH$_4^+$) is one of the main inorganic forms of nitrogen for plant nutrition. NH$_4^+$ is also an important nitrogen source for bacteria, fungi, and plants, but becomes toxic when it rises above certain levels [1–5].

Plants take up NH$_4^+$ with the help of specific transporters. AMT/MEP/Rhesus protein superfamily members function as electrogenic high-affinity NH$_4^+$ transporters [6–9]. Potassium (K⁺) channels can also mediate NH$_4^+$ uptake [10]. The Arabidopsis genome contains six AMT paralogs, four (AMT1;1, 1;2, 1;3, and 1;5) of which are together essential for NH$_4^+$ uptake [11, 12]. Unlike K⁺ channels, AMTs are highly selective for NH$_4^+$ and its methylated form, methylammonium (MeA) [6, 13]. In addition to their roles as transporters, AMTs can also function as receptors involved in the control of root growth and development, similar to the yeast MEP2 transceptor, which measures NH$_4^+$ concentrations to regulate pseudohyphal growth [14]. Recently, a ratiometric biosensor of NH$_4^+$ transporter activity, named AmTryoshka, which reports NH$_4^+$ transporter activity in vivo, was developed by inserting a cassette carrying two fluorophores into AMT1;3 [15]. Most organisms, including animals, plants, and even bacteria, are sensitive to high levels of NH$_4^+$. A sole supply of nitrogen as NH$_4^+$ is typically noxious [16]. In bacteria, the existence of highly effective detoxification mechanisms may have prevented the discovery of NH$_4^+$ toxicity. The actual mechanisms of NH$_4^+$ toxicity are not

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understood for any organism, but several hypotheses have been proposed: (i) pH effects—uptake and assimilation of NH$_4^+$ lead to acidification of the cytosol; (ii) membrane depolarization—NH$_4^+$ uptake depolarizes the membrane, thus high levels of NH$_4^+$ uptake could affect the capability of the cell to take up other nutrients; (iii) inhibition of electron flow in plastidic and mitochondrial membranes; (iv) increased production of reactive oxygen species that damage the cells [17, 18]; and (v) replacement of potassium as an enzyme cofactor, altering the catalytic properties and/or folding of enzymes that require K$^+$ [19, 20]. The increased NH$_4^+$ toxicity at low K$^+$ concentration strongly supports the K$^+$ replacement hypothesis [21]. Overaccumulation of ammonium can occur under various conditions, e.g., due to local placement of high doses by animals or by overfertilization. The concentrations of salts, and thus ammonium also rapidly increase during soil drying. To prevent the accumulation of NH$_4^+$ becoming toxic, the activity of AMTs is tightly regulated and likely based on feedback inhibition [22, 23]. Recent reports indicate that the phosphorylation of critical threonine (T460), which is triggered by NH$_4^+$ in the cytosolic C-terminus (CCT) of AMT1;1, leads to transport inhibition via allosteric regulation in the trimeric transporter complex [24, 25]. The AMT1;1 CCT, which serves as an allosteric switch, is highly conserved among the AMT homologs found in species ranging from bacteria to higher plants. Use of this allosteric regulation mechanism of AMT1;1 for feedback control allows plants to rapidly and efficiently block the uptake of NH$_4^+$ before levels become toxic. Yet, the full circuitry leading to NH$_4^+$-dependent phosphorylation of AMTs is not fully understood. We speculate that specific kinases are activated under conditions that lead to NH$_4^+$ accumulation.

Members of the CBL-interacting protein kinases [CIPK, also named SNF1-related kinases (SnRK)], which typically function together with members of the Calcineurin B-like protein (CBL) family, are known to regulate the activities of diverse types of transporters including the plasma membrane Na$^+$/H$^+$ exchanger SOS1 [26], the potassium channel AKT1 [27], magnesium transport [28], the nitrate transceptor CHL1 [29], the H$^+$/ATPase AHA2 [30], and several anion channels [31]. To examine whether CIPKs function as AMT regulators, we systematically screened for CIPKs able to affect AMT1;1 activity in Xenopus oocytes. In this study, we show that Arabidopsis CIPK15 acts as a negative regulator of AMT1;1 activity. CIPK15 directly interacted with AMT1;1 and inhibited AMT1;1 activity via phosphorylation of T460. This negative effect of CIPK15 on AMT1;1 activity was also observed by using an NH$_4^+$ transporter activity sensor-AmTryoshka1;3 LS-F138I, a ratiometric genetically encoded biosensor in yeast [15]. CIPK15 transcript levels increased in response to addition of external NH$_4^+$. Notably, compared to wild-type, cipk15 mutants showed higher $^{15}$NH$_4^+$ uptake and accumulated higher amounts of NH$_4^+$ and were hypersensitive to both NH$_4^+$ and MeA. Together, our data indicate that in the presence of elevated NH$_4^+$, CIPK15 inhibits AMT1;1 activity to prevent NH$_4^+$ toxicity.

**Results**

**CIPK15 can block the activity of AMT1;1 and 1;3**

To identify members of the CIPK family that can modulate AMT1;1 activity, two sets of mixtures of five CIPKs grouped according to their phylogenetic relationships [32] were co-expressed with AMT1;1 in Xenopus oocytes and AMT1;1 activity was recorded by two-electrode voltage clamping (TEVC) of Xenopus oocytes (Additional file 1: Figure S1). NH$_4^+$-induced inward currents were completely blocked by a mixture of CIPK2, 10, 15, 20, and 26, while the combination of CIPK3, 8, 9, 23, and 24 had no major impact on AMT1;1 activity (Additional file 1: Figure S1). The mixture of CIPK cRNAs was deconvoluted by testing individual CIPKs. During the first round of deconvolution, oocytes co-injected with equal amounts of AMT1;1 and CIPK15 cRNA showed strong inhibition of NH$_4^+$-induced inward currents of AMT1;1 (Fig. 1a, b). We therefore focused on CIPK15. Full inhibition of detectable AMT1;1-mediated NH$_4^+$-induced inward currents was also obtained when tenfold lower amounts of CIPK15 cRNA (0.5 ng) were co-injected with AMT1;1 (5 ng), a reduction of inward currents to below the detection level (Fig. 1c). Even at low CIPK15 cRNA levels, CBL1 did not lead to detectable activation of AMT1;1 activity (Additional file 1: Figure S2). The inhibition of AMT1;1 activity was not due to effects of CIPK15 co-expression on AMT1;1 levels as shown by protein gel blots (Additional file 1: Figure S3). Because we focused on CIPK15, we cannot exclude the possibility that other CIPKs may also affect AMT activity, in particular when co-expressed with CBLs. A ratiometric fluorescence biosensor for AMT1 activity, named AmTryoshka, which reports NH$_4^+$ transporter activity in vivo was previously engineered by inserting a cassette carrying two fluorophores into AMT1;3 [15]. AmTryoshka1;3 LS-F138I sensor shows a reduction in the 510 to 570 nm emission ratio when it is challenged with NH$_4^+$. Since the phosphorylation site T460 in AMT1;1 is conserved in AMTs (Additional file 1: Figure S4), we tested whether CIPK15 affects AMT1;3 activity by measuring the response of the ratiometric AMT activity sensor AmTryoshka1;3 LS-F138I in yeast (Fig. 2). Addition of NH$_4^+$ to yeast cells expressing the sensor led to a reduction in the relative 510 to 570 nm emission ratio. CIPK15, but not its kinase inactive form (CIPK15m), blocked NH$_4^+$-induced AmTryoshka LS-F138I responses (Fig. 2). Unlike CIPK15, CIPK19, which shares 52% identity with CIPK15, did not impair NH$_4^+$-
triggered AmTryishka1;3 LS-F138I response in yeast (Additional file 1: Figure S5). These data show that CIPK15 can exert its effect in different heterologous systems and can specifically inhibit both AMT1;1 and AMT1;3.

**NH₄⁺-induced CIPK15 mRNA accumulation**

In a screen of different heterologous systems for proteins that can affect AMT activity, we identified CIPK15 from Arabidopsis as a negative regulator. We therefore tested whether CIPK15 may be regulated at the transcriptional level or regulate the AMT activity by NH₄⁺. To test whether CIPK15 may be linked to NH₄⁺ nutrition, the expression of CIPK15 in wild-type root in response to the addition of 1 mM NH₄⁺ was examined. Similar to AMT1;1, CIPK15 mRNA levels increased by about 10-fold less than 1 h after adding NH₄⁺ (Fig. 3). These data indicate CIPK15 is NH₄⁺ inducible and plays roles in response to NH₄⁺ nutrition.

**CIPK15 can interact with different AMTs**

To test whether CIPK15 can interact with AMT1 isoforms, yeast split-ubiquitin interaction growth and β-galactosidase staining and filter assays were used [33]. AMTs (including 1;1, 1;2, 1;3, and 1;5) were fused to Cub (N-terminal ubiquitin domain fused to the artificial protease A-LexA-VP16 (PLV) transcription factor) and CIPK15 to NubG (N-terminal ubiquitin domain Ile-13 (NubI, positive control)) replaced by Gly; reduced affinity for Cub. Plasmids expressing AMTs, CIPK15, and controls (NubI and G) were expressed in yeast. Qualitative and quantitative assays (yeast interaction growth and β-galactosidase assays) demonstrated that CIPK15, but not CIPK19, can interact with AMT1;1 and several different AMTs (Fig. 4a–c, Additional file 1: Figure S6, control for Fig. 4a, and Additional file 1: Figure S7). The specificity of CIPK15-AMT1;1 interaction was further supported by split-fluorescent protein interaction assays in which different combinations of reconstitution of YFP fluorescence from AMT1;1 and CIPK15 or CIPK19 were used in N. benthamiana leaves (Fig. 4d and Additional file 1: Figure S8). Together, the protein interaction results in vitro and in vivo indicate that CIPK15 can interact specifically with several members of the AMT1 family.

**CIPK15 is necessary for NH₄⁺-triggered phosphorylation of T460 in AMT1**

AMTs contain multiple possible phosphorylation sites [34]. T460 in the conserved CCT, which immediately follows transmembrane spanning domain XI, plays a key role in the allosteric regulation of AMT1;1 [24]. To test whether CIPK15 is necessary for NH₄⁺-triggered phosphorylation of T460, we identified two cipk15 knockout
Fig. 2 CIPK15 inhibited AmTryoshka1;3 LS-F138I activity in yeast. 

**a** Schematic representation of AmTryoshka1;3 LS-F138I [15].

**b** CIPK15 reduced NH$_4^+$-triggered AmTryoshka1;3 LS-F138I [15] responses in yeast. AmTryoshka1;3 LS-F138I was co-expressed with control (vector only), CIPK15, and CIPK15m (inactive mutant). Results of normalized fluorescence ratio (normalized to buffer control = 1, λ$_	ext{exc}$ 440 nm, ratio = F$_{510/570}$) after addition of NH$_4$Cl as represented by box and whiskers (mean ± SE, n = 8). Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by Prism software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. $p$, significant change as shown in the figure (two-way ANOVA followed by Tukey’s post-test). PM, plasma membrane.
mutants and analyzed the AMT1:1 phosphorylation status (Additional file 1: Figure S9a-b). Growth of the mutants on MS media and in soil did not indicate any obvious phenotypic differences compared with the wild-type (Additional file 1: Figure S9c). Phospho-specific antibodies were used to test for CIPK15-mediated NH$_4^+$-triggered phosphorylation of T460 using protein gel blots. The knockout line AMT-qko (quadruple amt mutant) combines T-DNA insertions in AMT1;1, 1;2, 1;3, and 2;1, which was used as a negative control [35]. After 7 days of growth on MS media (high NH$_4^+$), AMT1;1 protein levels were not different in the cipk15 mutants and wild-type; however, the phosphorylation levels of AMT1 in the wild-type and cipk15 mutants were low (Additional file 1: Figure S10). In the wild-type, phosphorylation of AMT1 increased substantially within 1 h of exposure of N-starved plants to NH$_4^+$; AMT1 phosphorylation was undetectable in the cipk15 mutants (Fig. 5). We therefore conclude that CIPK15 is necessary for NH$_4^+$-triggered phosphorylation of AMT1.

**Fig. 3 NH$_4^+$-triggered CIPK15 mRNA accumulation.** qRT-PCR analyses of AMT1;1 and CIPK15 mRNA levels in roots after over 10 h after addition of 1 mM NH$_4^+$. Levels were normalized to UBQ10 (mean ± SE for four independent experiments (each experiment n > 50, total n > 200)); p, significant change for mRNA levels of AMT1;1 and CIPK15 at 1, 2, and 10 h compared to at 0 h (two-way ANOVA followed by Tukey’s post-test).

**cipk15 mutant shows high $^{15}$NH$_4^+$ uptake activity and NH$_4^+$ accumulation**

If CIPK15 is a key regulator that is necessary for T460 phosphorylation, one would predict that cipk15 mutants should accumulate more NH$_4^+$ and show elevated sensitivity to NH$_4^+$. To determine whether CIPK15 may be able to affect ammonium uptake and NH$_4^+$ toxicity in plants, cipk15 mutants were exposed to NH$_4^+$. Both cipk15 mutants were hypersensitive to NH$_4^+$ but not NO$_3^−$. After NH$_4^+$ pretreatment, cipk15 mutant seedlings accumulated higher amounts of NH$_4^+$ compared to the wild-type (Fig. 6a). Direct analysis of $^{15}$NH$_4^+$ uptake showed that cipk15 mutants imported more NH$_4^+$ relative to the wild-type (Fig. 6b). Together, our data showed that CIPK15 is necessary for NH$_4^+$-triggered inhibition of AMT1-mediated NH$_4^+$-uptake.

CIPK15 is a key factor for NH$_4^+$ tolerance in *Arabidopsis*

High levels of NH$_4^+$ negatively impact primary root growth [36–38]. To test whether high accumulation of NH$_4^+$ in cipk15 mutant and NH$_4^+$-induced phosphorylation of AMT1;1 by CIPK15 affects NH$_4^+$ sensitivity, root growth was analyzed in the presence or absence of NH$_4^+$.

Primary root length was not significantly different in the wild-type and qko mutants in media containing nitrate as the sole nitrogen source (Additional file 1: Figure S11). By contrast, primary root length of wild-type was dramatically reduced in media containing NH$_4$Cl or MeA (Fig. 7 and Additional file 1: Figure S11). Notably, cipk15 mutants were hypersensitive to NH$_4^+$, but not nitrate, as evidenced by shorter primary root length compared with wild-type and qko mutant plants (Fig. 7a and Additional file 1: Figure S12). Ammonium can be taken up via AMTs or K$^+$ channels. By contrast, the NH$_4^+$ analog methylammonium (MeA) is transported specifically via AMTs. cipk15 mutants were also hypersensitive to MeA, further supporting the hypothesis that CIPK15 is necessary for limiting AMT1;1 activity and that the effects observed for NH$_4^+$ can be related directly to the AMTs that contain the conserved domain including T460 (Fig. 7b). CIPK15 has been found to be involved in other processes or interactions with CBL1/4; however, there was no effect on AMT1;1 activity in Xenopus oocytes when co-expressed with CBL1 or a constitutively active form of CIPK19 with CIPK15 (Additional file 1: Figure S2), and no effect on primary root length in cbl4 and cipk19 mutants when they were exposed to 20 mM NH$_4$Cl and KNO$_3$ (Additional file 1: Figure S13) indicating that the effects observed with respect to ammonium toxicity are specific. Taken together, we conclude that CIPK15 activity is necessary for limiting NH$_4^+$ uptake by AMT1;1 when roots are exposed to NH$_4^+$ or MeA.
Discussion

Here, we identified the protein kinase CIPK15 as a key component in the NH$_4^+$-induced downregulation of ammonium uptake in Arabidopsis. CIPK15-mediated allosteric regulation of AMT1 activity may explain the observation that under field conditions, NH$_4^+$-uptake activity is negatively correlated with the external concentration of NH$_4^+$ concentrations in the soil [22].

Ammonium toxicity

Most plants are sensitive to high levels of NH$_4^+$ and supply with NH$_4^+$ alone typically causes symptoms of growth retardation [16]. Animals and fungi are sensitive to NH$_4^+$ as well, and recent work demonstrates that bacteria are also sensitive to NH$_4^+$. It is thus not surprising that ammonium uptake is under strict control and that the uptake rate is negatively correlated with the history of ammonium exposure [22]. Key questions are how toxic levels can be prevented, how the regulatory networks operate that limit ammonium accumulation, and how and where the cells sense ammonium, intracellularly or at the cell surface. The extreme conservation of the CCT in AMTs across kingdoms even in cyanobacteria and archaeabacteria as well as the dominant nature of mutations in the yeast homolog MEP1 piqued our interest and led to studies of the role of the CCT in AMT regulation [24, 39]. Genetic, biochemical and structural analyses have demonstrated that AMTs are
triple-barreled transporters that are allosterically regulated. Regulation is mediated by the CCT, which interacts with the respective neighboring subunits for transactivation [24]. A conserved residue, T460 in AMT1;1, T472 in AMT1;2, and T464 in AMT1;3, is phosphorylated in response to addition of NH$_4^+$ [40, 41]. We therefore hypothesized that either a receptor-like kinase or a cytosolic kinase is required for the feedback inhibition.

CIPK15 is necessary and sufficient for feedback inhibition
CIPKs are known to be involved in the regulation of the activity of diverse sets of transporters including AKT1, SOS1, NPF6.3, IRT1, etc., we therefore hypothesized that specific members of the CIPK family might be able to phosphorylate AMT1;1. To accelerate the screen, we co-expressed sets of five CIPK genes together with AMT1;1 and monitored AMT activity using TEVC. Based on our functional interaction screen assays, we identified and deconvoluted one of the mixtures that led to reduced AMT1;1 activity. CIPK15 by itself was sufficient to substantially inhibit AMT1;1 activity. The inhibition effect on AMT1;1 activity was still obtained when lower amounts of CIPK15 cRNA were co-injected with AMT1;1 in oocytes, and CBL1 did not cause the activation of AMT1;1 activity in oocyte. We cannot exclude the possibility that some AMT1;1 activity remains even in the inhibited state in the oocyte system, but the activity was below the detection limit. Commercial oocytes are often lower quality compared to oocytes isolated freshly from locally held frogs, thus it is conceivable that experiments in which higher AMT activity can be detected CIPK15 may also reveal remaining AMT1 activity. However, our data demonstrate that, when co-expressed with AMT1;1 in oocytes, CIPK15 inhibits AMT1;1 activity. Moreover, upon functional interaction assay in yeast, CIPK15 also inhibited NH$_4^+$-induced fluorescence change in the transport activity biosensor AmTryoshka1;3, indicating that CIPK15 can affect the activity of multiple AMT paralogs. The effect of CIPK15 on the AMTs is likely direct and specific, since CIPK15, but not CIPK19, can interact with AMT1;1 or multiple AMTs and tune AMT activities. Importantly, mutant analyses demonstrate that CIPK15 is also necessary for NH$_4^+$-triggered AMT1;1 phosphorylation (T460). cipk15 mutants took up and accumulated more NH$_4^+$ and were hypersensitive to NH$_4^+$ and the analog MeA. The MeA sensitivity of cipk15 mutants intimates that the effects observed with respect to ammonium toxicity are due to inhibition of AMT activity, since MeA is transported by AMTs but not by potassium channels. Since CIPK15 is a factor produced in the cytosol, the action of the kinase is intracellular. This work, therefore, identifies the key kinase for AMT regulation, which represents a major step forward for the elucidation of the full regulatory circuit. AMT1;2 also plays an important role in NH$_4^+$ uptake. Data from other groups may indicate that in oocytes, AMT1;2 mediates larger ammonium-induced inward currents when compared to AMT1;1 [42]. It remains open, whether the larger currents are due to different quality of oocytes from in house versus commercial facilities. The next experiments will need to address where and how NH$_4^+$ is sensed. CIPK15 and the AMTs may be useful tools to unravel the remaining steps in the regulatory circuitry.

![Fig. 5 AMT1;1-T460 phosphorylation is reduced in cipk15 mutant plants. Plant seedlings were germinated and grown for 7 days in half-strength MS medium with 5 mM KNO$_3$ as the sole nitrogen source, then starved for 2 days in half-strength MS medium without nitrogen. Seedlings were treated with 1 mM NH$_4$Cl for 1 h, membrane fractions were isolated and probed with anti-AMT1-P antibodies (a) and anti-AMT1;1 antibodies (b) [25]. Ponceau S staining served as a loading control. Quantification of phosphorylation of AMT1-P levels normalized to Ponceau S staining and relative to wild-type shown in c. Corresponding data and replications were obtained in three independent experiments. Data (c) are the mean ± SD for three experiments. p, significant change compared to wild-type as shown in figure (two-way ANOVA followed by Tukey’s post-test).](image-url)
The relative role of CIPK15 and CIPK23 in AMT regulation

Recent work has indicated that another CIPK, namely CIPK23, plays a role in the regulation of AMT1;1 and AMT1;2 [42]. The authors showed that CIPK23 can interact with AMT1;1 and AMT1;2 but did not observe an interaction with AMT1;3. Here, we identified an interaction between CIPK15 and AMT1;1 by using split-ubiquitin yeast two-hybrid assays in yeast, split-fluorescent protein interaction assays in *N. benthamiana* leaves, and functional interaction by TEVC in *Xenopus* oocytes. Interactions of CIPK15 with AMT1;2 and AMT1;3 were also identified by using split-ubiquitin yeast two-hybrid in yeast, and a functional interaction of CIPK15 with AMT1;3 was validated with the help of a ratiometric NH$_4^+$ transporter activity reporters in yeast. Consistent with the conservation of the domain surrounding the phosphorylation site (T460 in AMT1;1), the protein interaction and functional assay results indicate that CIPK15 likely affects the activity of all three AMT1 paralogs. According to public transcriptome databases (e.g., TAIR, Genevestigator), CIPK23 and CIPK15 appear to be expressed in AMT1;1-expressing tissues. Here, we also found that CIPK15 and CIPK23 mRNA increased in response to NH$_4^+$ addition (Additional file 1: Figure S14). Notably, CIPK15 mRNA accumulation triggered by 1 mM NH$_4^+$ was about three and half-fold higher relative to the CIPK23 mRNA accumulation; absolute levels of CIPK15 are similar after ammonium addition compared to CIPK23. Consistent with the interaction, coexpression of CIPK23 in the presence of CBL1 led to about a twofold lower current for AMT1;2 in *Xenopus* oocytes, while CIPK15 led to essentially complete loss of detectable AMT1;1-mediated currents. The data from the two labs are not directly comparable, since Straub et al. observed larger currents when analyzing the effect of CIPK23 on AMT1;2. AMT1;3 activity was also impaired by CIPK15 as shown using AmTryoshka1;3. While the experiments were not performed side by side, these data may indicate that CIPK23 plays a different and less prominent role as compared to CIPK15. In *cipk23* mutants, AMT1;1-GFP phosphorylation was reduced by ~20% for AMT1;1 and ~40% for AMT1;2. In comparison, *cipk15* mutants completely lost detectable AMT phosphorylation. In *cipk23* mutants, the shoot dry weight was reduced, and they showed higher $^{15}$NH$_4^+$ uptake in the presence of ammonium relative to the wild-type. However, *cipk23* mutants displayed no difference regarding hypocotyl length when exposed to 20 mM $^{15}$NH$_4^+$. Well-characterized CIPK15-interactors, CBLS, did not have an apparent effect on AMT1;1 activity in *Xenopus* oocytes, nor was NH$_4^+$ toxicity in *cbl* mutants affected. Taken together, the data indicate that multiple CIPKs can affect AMT activity with different efficacy, possibly different tissue specificity, different specificity for AMT paralogs, and with differing dependence on CBLS.

Conclusions

Taking the results together, this work identified a key component in the NH$_4^+$ feedback inhibition network, namely the protein kinase CIPK15, which directly interacts with AMTs to phosphorylate the conserved threonine in their C-terminus to adjust ammonium uptake and dependence on the external NH$_4^+$ concentration. The remaining open questions in the field are how the plant senses the ammonium concentration and how it activates CIPK15. CIPK15 has been reported to be involved in the ABA signaling pathway and phosphorylation of ERF7, an APETALA2/EREBP-type transcription factor [43, 44], and more recently, CIPK32 was shown to play a role in the regulation of AMT activity; it will be interesting to explore the ABA response and the interrelationship between CIPK32 phosphorylation of residues downstream of T460 and the two CIPKs [45].

Methods

Plant materials and treatments

Experiments were performed with *Arabidopsis thaliana* ecotype Col-0. The knockout lines of AMT-*qko*, the quadruple AMT which carry T-DNA insertions in AMT1;1, 1;2, 1;3, and 2;1, cbl4 (At5g24270, SALK_113101), and *cipk19* (At5g45810, SALK_044735) have been previously described [35, 46, 47]. Plant growth conditions have also been previously described [25] and were used here with minor modifications. Arabidopsis seeds were surface sterilized and germinated on half-strength modified Murashige Skoog medium (MS), nitrogen-free salts (Phytotechlab, M407) with 5 mM KNO$_3$ as the sole nitrogen source, 0.5% [w/v] sucrose, and 1% [w/v] agar, pH 5.8 [KOH] on vertical plates. For qRT-PCR, protein blots, NH$_4^+$ content, and $^{15}$N-labeled uptake assays, seedlings (7 days after germination) were transferred to the half-strength MS medium lacking nitrogen for 2 days, and then, seedlings were transferred to half-strength MS medium supplemented with NH$_4$Cl, $^{15}$NH$_4$Cl or NO$_3^-$ according to the concentrations indicated in the respective figure legends. Roots were collected and frozen in liquid nitrogen. Seedlings were incubated in a 16/8 h light/dark period at 22 °C. For NH$_4^+$ content, seedlings were collected after being starved for 2 days, or after treatment with 1 mM NH$_4$Cl, and 1 mM KNO$_3$ for 1 h. For $^{15}$N-labeled uptake assays, seedlings were collected after being starved for 2 days (1 mM $^{15}$NH$_4$Cl was used for the last 15 mins for $^{15}$N-labeling), or after treatment with 1 mM NH$_4$Cl, and 1 mM KNO$_3$ for 1 h (1 mM $^{15}$NH$_4$Cl was used for the last 15 mins for $^{15}$N-labeling). $^{15}$N-labeled seedlings were then dried for 2–3 days at 65 °C and further analyzed by Thermo Finnigan Delta plus XP IRMS (ThermoFisher Scientific). For primary root length determination, seedlings (3 days after germination) were transferred to half-
strength MS medium with nitrogen-free salts, containing KNO₃, NH₄Cl, or methylammonium (MeA) as nitrogen sources at the concentrations indicated in the figures, and grown for another 1 to 5 days. Seedlings were scanned on a flatbed scanner, and primary root length was measured using NIH ImageJ software (imagej.nih.gov).

Characterization of T-DNA insertion mutants
Col-0, CIPK15 (SnRK3.1) (At5g01810) T-DNA insertion lines cipk15-1 (SALK_203150) and cipk15-2 (GK6043806) were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/abrc/). T-DNA insertions in CIPK15 were confirmed by PCR analysis and sequencing using the T-DNA left border primer (5′-TGGTTCACTAGTGCGCCATCA) and CIPK15 F (5′-TCTTCTGTTGGTGGACACG) and R (5′-TGGGTAGTTCAGTGTGTCACC) primers. H3G1 (At4g40040) was used as the loading control (H3G1, forward primer: 5′-AACCACTGGAGGAGTCAAGA-3′; reverse primer: 5′-CAATTAAGCACGTTCCTCCT-3′).

Real-time qRT-PCR analyses
Real-time qRT-PCR was performed as previously described [29]. In brief, template cDNA samples were prepared using 4 μg of total RNA and the Improm-II reverse transcription system (Promega). Primers were designed to have a Tₘ of ~60 °C and to produce PCR products of ~200–400 base pairs. Expression levels in each experiment were first normalized to the expression of Ubiquitin10 measured in the same cDNA samples (AMT1;1, forward primer: 5′-ACGACATTATCAGTCGCG; reverse primer: 5′-CTGTCCTGTGATTAAAG; CIPK15, forward primer: 5′-GGCTACGCATCTGACT; reverse primer: 5′-CTTGACAGCTTCCTAGTAC; CIPK23, forward primer: 5′-CTCTGTTGCTATGAGGCAG; reverse primer: 5′-TGGATATCAGTCTGAATCC, and Ubiquitin10, forward primer: 5′-CTCTTCAACAGAGACGAG).

Quantification of ammonium levels in plants
NH₄⁺ content was determined colorimetrically at 410 nm after reaction with Nessler’s reagent [48]. In brief, 500 mg of fresh matter was added to 1 ml of deionized water and shaken for 1 h at 45 °C. Samples were centrifuged at 15,000 g for 20 min. Ammonium content was determined on 50 μl of the supernatant using 1 ml of Nessler’s reagent (Merck) and quantified by using a standard curve and expressed as μmol g⁻¹ FW.

Split-ubiquitin yeast two-hybrid assays
The split-ubiquitin yeast two-hybrid assay was as described previously [33]. In brief, ORFs of interest were cloned in frame with either the C-terminal (Cub) of TMBV4 vector or N-terminal (NubG; wild-type l-13 replaced by G) domain of ubiquitin in pDL2Nx vector, and then introduced into yeast strains AP4 and AP5 by the lithium acetate method [49]. For interaction growth
assays, yeast was transformed with plasmids containing AMT1s-Cub, CIPK15-Nub, CIPK19-Nub, NubI, or NubG. Colonies were picked and cells were serially diluted four-fold and grown for 2 days in either SD-Trp Leu (control) or SD-Trp Leu His (for interaction). β-galactosidase (β-gal) activity was determined using filter assays, X-gal staining, and quantitative β-gal assays [50]. For filter assays, cells were streaked on filter paper, briefly frozen in liquid nitrogen, defrosted, and placed in Petri dishes filled with 0.5% agarose containing 35 mM β-mercaptoethanol (v/v) and 1.5 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Sigma). For X-gal staining, yeast co-expressing bait and prey fusions were streaked onto minimal medium lacking leucine and tryptophan and onto media plates supplemented with X-gal. For quantitative β-gal assays, cells were grown in minimal medium lacking leucine and tryptophan at 30 °C overnight to OD$_{600}$ of ~ 0.75, centrifuged, and washed in 1 ml Buffer Z, 113 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, and 1 mM MgSO$_4$). To perform the assay, 300 μl Buffer Z was added to the pellets and vortexed before lysing cells by 3 freeze-thaw cycles. Lysate (100 μl) was added immediately to 700 μl Buffer Z containing 0.27% β-mercaptoethanol before the addition of 160 μl of 4 mg/mL 2-nitrophenyl-beta-D-galactopyranoside (ONPG) in buffer Z. The lysate was incubated at 30 °C for 180 min. Reactions were stopped by adding 0.4 ml of 0.1 M Na$_2$CO$_3$. Samples were centrifuged, and OD$_{420}$ of the supernatant was measured. For each prey-bait combination, five independent colonies were taken and the results were averaged.

**Split-fluorescent protein interaction assays in tobacco leaves**

Potential AMT1;1 and CIPK interactions were tested in a tobacco transient expression system using a modified split-fluorescent protein assay as previously described [51]. In brief, AMT1;1 and CIPKs were PCR amplified, cloned into the Gateway entry vector pENTR/D/TOPO, and then recombined into the Gateway binary destination vectors pXNGW, pNXGW, pCXGW, and pXCWG using LR clonase (Invitrogen). Each protein was independently tagged with cCFP and nYFP at either the N or C terminus. The binary vector backbone was derived from pZP312, which contains a single 35S cauliflower mosaic virus promoter and terminator derived from pRT100. The binary constructs were further introduced into A. tumefaciens strain GV3101. Cell density was adjusted with infiltration buffer to OD$_{600}$ ~ 0.5. Agrobacteria harboring the Tomato Bushy Stunt Virus P19 silencing suppressor were co-infiltrated to reduce gene silencing. Aliquots (0.5 ml) of Agrobacterium cells carrying a split-fluorescent protein fusion construct and P19 constructs were mixed. A syringe was used to infiltrate the mixture into the abaxial side of N. benthamiana leaves. Plants were incubated in a growth chamber at 22 °C, with a 16-/8-h day/night cycle for 36 to 48 h. Re-constitution of yellow fluorescent protein (YFP) fluorescence, chlorophyll, and bright field images in the transformed N. benthamiana leaves were recorded using confocal fluorescence microscopy (LSM780; Carl Zeiss).

**Extraction of membrane fractions and protein gel blot analyses**

For membrane preparation, roots or oocytes were ground in liquid nitrogen and resuspended in buffer containing 250 mM Tris-Cl, pH 8.5, 290 mM sucrose, 25 mM EDTA, 5 mM β-mercaptoethanol, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.53 mM Complete Protease Inhibitor Cocktail (Sigma-Aldrich), and 0.53 mM PhosStop Phosphatase Inhibitor Cocktail (Roche Applied Science). After centrifugation at 10,000g for 15 min, supernatants were filtered through Miracloth (Calbiochem) and recentrifuged at 100,000g for 45 min. The sediment containing the microsomes was resuspended in storage buffer [400 mM mannitol, 10% glycerol, 6 mM MES/Tris, pH 8, 4 mM DTT, 2 mM PMSF, and 13 mM phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich)]. Proteins were denatured in loading buffer (62.5 mM Tris-Cl, pH 6.8, 10% [v/v] glycerol, 2% [w/v] SDS, 0.01% [w/v] bromophenol blue, and 1% PMSF), incubated at 37 °C for 30 min with or without 2.5% [v/v] β-mercaptoethanol at 0 °C, and then electro-phoresed in 10% SDS polyacrylamide gels (Invitrogen) and transferred to polyvinylidene fluoride membranes. Proteins were detected using the anti-AMT1;1 antibody or the anti-P-AMT1;1 T460 antibody [25]. Blots were developed using an ECL Advance Western Blotting Detection Kit (Amersham). Protein and phosphorylation levels were measured using ImageJ software.

**Two-electrode voltage clamp of AMT1;1 in Xenopus oocytes**

Two-electrode voltage clamp (TEVC) measurements were performed in Xenopus oocytes as previously described [52]. In brief, ORFs of AMT1;1, CBL1, the constitutively active CIPK19-CA (Thr186 to Asp), and the 10 CIPKs (CIPK2, 3, 8, 9, 10, 15, 20, 23, 24, 26 and CBL1, 4, kind gift from Jörg Kudla, Münster, Germany) in two pools of 5 in Gateway pDONR221 donor vector were further cloned into pOO2-GW via LR reactions of basic of Gateway Cloning Protocols (https://www.thermofisher.com/tw/en/home/life-science/cloning/gateway-cloning/protocols.html) using LR Clonase II enzyme (Invitrogen). For in vitro transcription, pOO2GW plasmids were linearized with MluI or another suitable restriction enzyme. Capped cRNA was in vitro transcribed by SP6 RNA polymerase using mMESSAGE mMACHINE kits (Ambion, Austin, TX). Xenopus laevis oocytes were obtained from Ecoscyte Bio Science (Austin,
TX). Oocytes were injected with distilled water (50 nl as control) or cRNA from AMT1;1, CIPKs, CBL1, CBL4, or CIPK19-CA (0.5 ng to 50 ng of cRNA as indicated in figure legends in 50 nl) using a robotic injector (Multi Channel Systems, Reutlingen, Germany) [53, 54]. Cells were kept at 16 °C for 2–4 days in ND96 buffer containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.4, and gentamycin (50 μg/μl). Electrophysiological analyses were typically performed 2–3 days after cRNA injection as previously described [52]. Typical resting potentials were about −40 mV. For current (I)-voltage (V) curves, measurements were recorded from oocytes that were first clamped at −40 mV followed by a step protocol to determine voltage dependence (−20 to −200 mV for 300 ms; in −20 mV increments). The current-voltage relationships were measured by the TEVC Roboocyte system (Multi Channel Systems) [53, 55].

Fluorimetric analyses of AmTryoshka LS-F138I with CIPKs in yeast
Fluorimetric analyses were performed in yeast as previously described [15]. In brief, CIPK15, CIPK19, and CIPK15m (K41N, an inactive form) were introduced into yeast expressing AmTryoshka1;3 LS-F138I. Vector only was used as the control. Cells were analyzed in 96-well, flat-bottom plates (Greiner Bio-One, Germany). Steady-state fluorescence was recorded using a fluorescence microplate reader (Infinite, M1000 pro, Tecan, Switzerland) in bottom-reading mode using 7.5 nm bandwidth and a gain of 100. The fluorescence emission spectra (λ_{exc} 440 or 485 nm; λ_{em} 510 or 570 nm) were background-subtracted using yeast cells expressing a non-fluorescent vector control.

Supplementary Information
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Additional file 1: Figure S1. Screen for CIPK effects on AMT1;1 activity in Xenopus oocytes. Figure S2. The activity of AMT1;1 co-expressed with CIPKs or CBL in Xenopus oocytes. Figure S3. Protein gel blots for AMT1;1
protein level in Xenopus oocytes as control for Fig. 1. Figure S4: Alignment of the TMH XI and C terminus of five members of the AMT1 family. Figure S5: CIPK19 has no effect on AmTryoshka1;1 LS-F138I activity in yeast. Figure S6: Split-fluorescent protein interaction assay for AMT1;1 and CIPKs in Nicotiana benthamiana leaves. Figure S9: Arabidopsis T-DNA insertion mutants of cipk15-1 and cipk15-2. Figure S10: Protein gel blots for AMT1;1 protein and AMT-P phosphorylation levels in wild-type and cipk15 mutant plants under half-strength MS medium. Figure S11: Primary root length of control (wild-type) and qko mutant on half-strength MS medium containing NH4Cl, KNO3, or MeA. Figure S12: Primary root length of Col-0, qko, and cipk15 mutant plants on media containing KNO3 as sole nitrogen source. Figure S13: cipk19 and cipk19 mutants do not show ammonium hypersensitivity. Figure S14: CIPK23 mRNA accumulation by NH4+.

Additional file 2. Individual values for figures where the number of independent replicates is less than 6 (n<6). Each sheet in the Excel-file is named by the figure the data represent.

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Authors’ contributions
Conceptualization, C.-H.H. and W.B.F.; methodology, C.-H.H., H.-Y.C., Y.-N.C., and H.-W.Y.; investigation, C.-H.H., H.-Y.C., Y.-N.C., H.-W.Y. and Z.-T.L.; writing, C.-H.H. and W.B.F.; supervision, C.-H.H. The work was initiated by C.-H.H. in Conceptualization, C.-H.H. and W.B.F.; methodology, C.-H.H., H.-Y.C., Y.-N.C., and H.-W.Y.; writing, C.-H.H. and W.B.F.; supervision, C.-H.H. and W.B.F.; and C.-H.H. The authors declare no competing interests.

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