

# Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation

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**To counteract fluctuating nutrient environments, plants have evolved high- and low-affinity uptake systems. These two systems were traditionally thought to be genetically distinct, but, recently, two *Arabidopsis* transporters, AtKUP1 and CHL1, were shown to have dual affinities. However, little is known about how a dual-affinity transporter works and the advantages of having a dual-affinity transporter. This study demonstrates that, in the case of CHL1, switching between the two modes of action is regulated by phosphorylation at threonine residue 101; when phosphorylated, CHL1 functions as a high-affinity nitrate transporter, whereas, when dephosphorylated, it functions as a low-affinity nitrate transporter. This regulatory mechanism allows plants to change rapidly between high- and low-affinity nitrate uptake, which may be critical when competing for limited nitrogen. These results demonstrate yet another regulatory role of phosphorylation in plant physiology.**

**Keywords:** affinity/*Arabidopsis*/nitrate/phosphorylation/transporter

## Introduction

Plants have a remarkable ability to adapt and grow in soils containing widely different levels of ions and nutrients. Since the early studies of Epstein in the 1950s (Epstein, 1972), it has been known that plants have evolved two types of system for nutrient uptake, a high-affinity system and a low-affinity system. The high capacity low-affinity uptake system takes up nutrients when the external substrate concentration is high (e.g. >0.5 mM for nitrate), while the high-affinity system can scavenge substrate at low concentrations (<0.2 mM for nitrate). The saturation kinetics of ion uptake for the two systems have been interpreted as evidence for at least two transporters with distinct affinities, and this idea was further supported by the identification of distinct genes for the respective systems. For example, a model based on the observed biphasic kinetics of potassium influx proposes that high-affinity active uptake is mediated by proton/potassium symporters, while low-affinity passive uptake is mediated by inward-rectifying potassium channels (Kochian and Lucas, 1988; Maathuis *et al.*, 1997). Indeed, both proton-coupled potassium transporter genes (HAK/KUP family) and potassium channel genes (AKT/KAT family) have been isolated in higher plants (Anderson *et al.*, 1992;

Sentenac *et al.*, 1992; Santa-Maria *et al.*, 1997). In contrast to potassium uptake, both the high- and low-affinity uptake of negatively charged nitrate are proposed to be mediated by proton-coupled symporters (Glass and Siddiqi, 1995), and, consistent with this, two families of nitrate transporter genes, *NRT1* and *NRT2*, which share no sequence similarity with each other, have been identified in higher plants (Tsay *et al.*, 1993; Trueman *et al.*, 1996) and have been thought for the past decade to be responsible for low- and high-affinity nitrate uptake, respectively.

In contrast to this model in which high- and low-affinity uptake are mediated by different genes, a knock-out mutant of the *AKT1 Arabidopsis* potassium channel, which was originally thought to be responsible for low-affinity potassium uptake, was also found to be defective in high-affinity potassium uptake (Hirsch *et al.*, 1998). Likewise, the *Arabidopsis* potassium transporter, AtKUP1, has also been shown to be a dual-affinity transporter (Fu and Luan, 1998; Kim *et al.*, 1998); when expressed in yeast, it exhibits a  $K_m$  of ~44  $\mu$ M for the high-affinity phase of uptake and ~11 mM for the low-affinity phase (Fu and Luan, 1998). At almost the same time, it was found that the *Arabidopsis* low-affinity nitrate transporter mutant *chl1 (nrt1)* is also defective in high-affinity nitrate uptake (Wang *et al.*, 1998; Liu *et al.*, 1999). Using the *Xenopus* oocyte expression system, it was further demonstrated that CHL1 functions as a dual-affinity nitrate transporter with a  $K_m$  of ~50  $\mu$ M for the high-affinity phase of uptake and ~4 mM for the low-affinity phase (Liu *et al.*, 1999). These discoveries of dual-affinity uptake by a single transporter raised the question of whether the kinetic properties of other characterized transporters had been examined over a wide enough range of concentrations to rule out the possibility that they also were dual-affinity transporters, especially since *chl1* had been considered as a low-affinity nitrate uptake mutant for >20 years (Doddema and Telkamp, 1979; Tsay *et al.*, 1993; Huang *et al.*, 1996; Touraine and Glass, 1997).

Numerous proteins are known to have dual functions. Many use spatially distinct domains for each function. For example, the TRP family uses an N-terminal domain for its channel activity and a C-terminal domain for enzyme activity (kinase or ADP-ribose pyrophosphatase; Cahalan, 2001), the enzyme activity being required for channel activity (Perraud *et al.*, 2001; Runnels *et al.*, 2001). In contrast, in some dual-function proteins, the mode of action is modulated by post-translational modifications or ligand binding. For example, depending on which amino acid residue (threonine 34 or threonine 75) is phosphorylated, DARPP-32, a dopamine- and cyclic AMP-regulated phosphoprotein, functions as either a kinase inhibitor or a phosphatase inhibitor (Bibb *et al.*, 1999). Calmodulin modulation of the P/Q-type ( $\alpha$ 1A) calcium channel is another prime example, as binding of calcium to the

N-terminal or C-terminal lobe of calmodulin results, respectively, in inactivation or activation of the P/Q-type calcium channel (DeMaria *et al.*, 2001).

Dual-function proteins may not have evolved for an economic reason, i.e. to minimize the size of the genome, since gene duplication and functional redundancy are quite common in higher organisms, e.g. 60% of the *Arabidopsis* genome consists of duplicated segments (The Arabidopsis Genome Initiative, 2000), but rather because of some advantage of a regulatory switch and interplay between two distinctive modes of activities. It is therefore important to study the underlying operating mechanisms of dual-affinity transporters and their physiological significance. In the present study on CHL1, we have elucidated a possible mechanism and identified a novel process of post-transcriptional regulation for plant nutrient uptake.

## Results

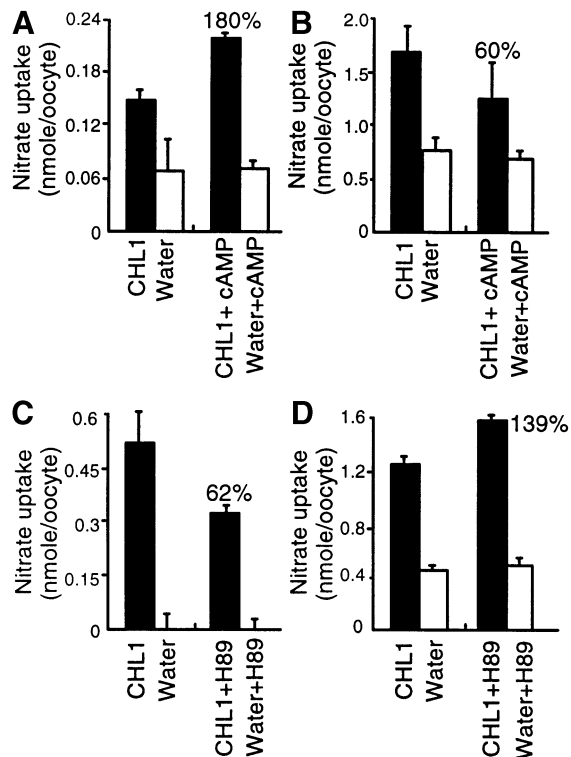
### High- and low-affinity nitrate uptake activities of CHL1 are differentially regulated by PKA activators or inhibitor

Since phosphorylation is known to modulate ion channel activity (Esguerra *et al.*, 1994; Wang *et al.*, 1996; Beguin *et al.*, 1999; Gong *et al.*, 1999; Schiff *et al.*, 2000), we speculated that it might also play a role in regulating CHL1-mediated uptake activity. We therefore examined nitrate uptake by *CHL1* cRNA-injected *Xenopus* oocytes in which cAMP-dependent protein kinase (PKA) activity was either increased or reduced by cAMP agonists or a PKA inhibitor.

When oocytes were treated with cAMP agonists [a mixture of 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP), forskolin and 3-isobutyl-1-methylxanthine (IBMX)] to enhance PKA activity, the nitrate uptake activity of *CHL1*-injected oocytes in medium containing 150  $\mu$ M nitrate (high-affinity concentration) increased to ~180% of that in the untreated control (Figure 1A), while that measured using 10 mM nitrate (low-affinity concentration) decreased to ~60% of untreated control levels (Figure 1B). Conversely, when *CHL1*-injected oocytes were treated with the PKA inhibitor H89, the uptake activity measured using 150  $\mu$ M nitrate decreased by ~40% (Figure 1C), while that measured using 10 mM nitrate increased by ~40% compared with control levels (Figure 1D). Similar results were obtained using another two batches of oocytes isolated from two other toads (Table I). These data suggest that phosphorylation activates the high-affinity nitrate transport activity of CHL1 and inhibits the low-affinity transport activity.

### CHL1 protein can be phosphorylated on threonine residues

To determine whether the CHL1 protein could be phosphorylated, a plasma membrane-enriched fraction was purified from *CHL1*- or water-injected oocytes, then part of the sample was subjected to immunoblot analysis using anti-CHL1 antibodies (Figure 2A), while the rest of the sample was incubated with [ $\gamma$ - $^{32}$ P]ATP and cAMP-dependent protein kinase catalytic subunit, immunoprecipitated with anti-CHL1 antibodies, then electrophoresed and an autoradiogram made from the gel (Figure 2B). The results showed that, compared with the water-injected

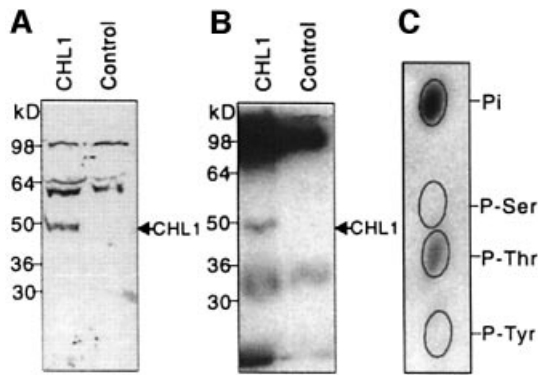


**Fig. 1.** Effect of cAMP agonists and a PKA inhibitor on high- and low-affinity nitrate uptake in *CHL1* cRNA-injected oocytes. (A and B) Effect of cAMP agonists on the high- (A) and low- (B) affinity nitrate uptake activity of *CHL1*-expressing oocytes. Water- or *CHL1*-injected oocytes were pre-incubated for 90 min in ND-96 medium (Roche and Treisman, 1998) alone or in ND-96 medium containing a mixture of 0.5 mM 8-Br-cAMP, 50  $\mu$ M forskolin and 0.5 mM IBMX, then uptake was measured. (C and D) Effect of the PKA inhibitor H89 on the high- (C) and low- (D) affinity nitrate uptake activity of *CHL1*-expressing oocytes. As in (A) and (B), except that pre-incubation was in ND-96 medium or in ND-96 medium containing 50  $\mu$ M H89. High-affinity nitrate uptake was measured using 150  $\mu$ M  $\text{NO}_3^-$  and low-affinity uptake using 10 mM  $\text{NO}_3^-$ ; oocytes were incubated with the nitrate solution for 90 min, then the amount of nitrate retained in the oocyte (low-affinity uptake) or depleted from the solution (high-affinity uptake) was determined by HPLC (Liu *et al.*, 1999). Every data point represents the mean of three measurements, each based on five oocytes from a single batch; the error bar represents the standard error. Similar results were obtained using another two batches of oocytes isolated from two different toads (see Table I).

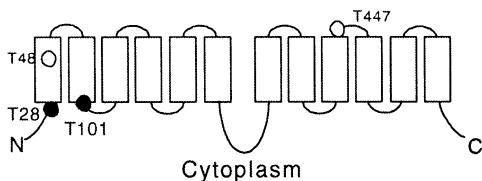
**Table I.** Nitrate uptake activities of *CHL1*-injected *Xenopus* oocytes treated with PKA activators or a PKA inhibitor

cAMP agonists	Percentage of control activity measured at	
	150 $\mu$ M	10 mM
Exp. 1	180	60
Exp. 2	206	41
Exp. 3	223	12
H89	Percentage of control activity measured at	
	150 $\mu$ M	10 mM
Exp. 1	62	139
Exp. 2	31	158
Exp. 3	0	185

The three experiments were performed in triplicate (samples of five oocytes) on three different batches of oocytes from different toads.



**Fig. 2.** Phosphorylation of CHL1 at threonine residues. (A) Immunoblot analysis of *Xenopus* oocyte membrane proteins probed using anti-CHL1 antibodies. Plasma membrane-enriched fractions, isolated from CHL1- or water-injected oocytes (Maurel *et al.*, 1995), were separated by SDS-PAGE for immunoblot analysis using anti-CHL1 antiserum. (B) Autoradiogram of *in vitro* phosphorylated oocyte membrane proteins immunoprecipitated using anti-CHL1 antibodies. The same fractions used in (A) were phosphorylated *in vitro* in the presence of [ $\gamma$ - $^{32}$ P]ATP (NEN Life Science Products) and cAMP-dependent kinase catalytic subunit (New England Biolabs). (C) Phosphoamino acid analysis of *in vitro* phosphorylated CHL1. The 50 kDa phosphorylated peptide on gel (B) was eluted, hydrolyzed and subjected to thin-layer electrophoresis at pH 3.5 (Xu *et al.*, 1996). The ovals show the positions of the non-radioactive standards, phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr).



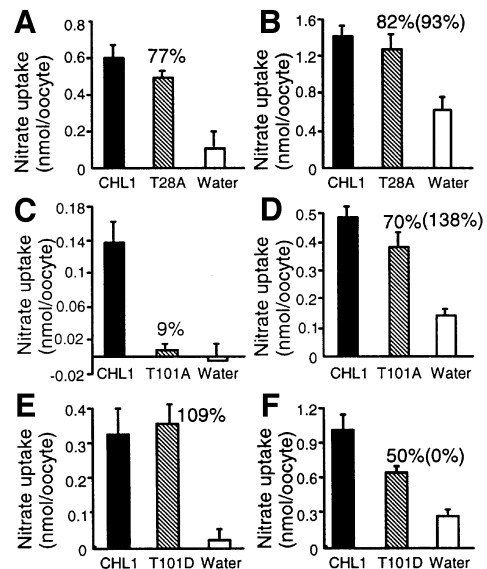
**Fig. 3.** Putative membrane topology and phosphorylation sites of CHL1. The black circles represent the consensus phosphorylation sites facing the cytoplasm and the white circles those buried in the membrane or on the extracellular surface.

control, CHL1-injected oocytes contained an additional band that could be phosphorylated *in vitro* (Figure 2B) and had the same mobility as CHL1 detected in immunoblots (Figure 2A).

When this 50 kDa phosphorylated protein was eluted and subjected to phosphoamino acid analysis, the results showed that it was phosphorylated on threonine residues (Figure 2C). The CHL1 protein sequence contains four putative phosphorylation sites (RXXT/S), all of which use threonine as the phospho-acceptor (Figure 3). In the widely accepted membrane topology model for CHL1 and related transporters (Hagting *et al.*, 1997; Covitz *et al.*, 1998), two of these four putative phosphorylation sites, T28 and T101, face the cytoplasm and can probably be phosphorylated. These two sites were therefore subjected to site-directed mutagenesis analysis.

#### **Mutation at threonine 101 affects the high- and low-affinity nitrate uptake activities of CHL1**

Threonines 28 and 101 were separately mutated to alanine and the mutants tested in nitrate uptake studies. When compared with *Xenopus* oocytes expressing wild-type



**Fig. 4.** Nitrate uptake activities and kinetic analyses using wild-type CHL1-, T28A-, T101A-, or T101D-injected oocytes. Nitrate uptake in oocytes injected with cRNAs encoding wild-type CHL1 or mutants T28A (A and B), T101A (C and D), or T101D (E and F). (A, C and E) High-affinity nitrate uptake activity determined by incubating oocytes with 150  $\mu$ M  $\text{NO}_3^-$  for 1.5 h; (B, D and F) nitrate uptake activities determined by incubating oocytes with 10 mM  $\text{NO}_3^-$  for 1.5 h. The numbers above the striped bars indicate the activity expressed as a percentage of wild-type CHL1 activity after subtraction of background activity (water-injected oocytes), while the numbers in parentheses show the actual low-affinity nitrate uptake activity calculated by subtracting the contribution of the high-affinity component from the 10 mM nitrate uptake activity.

CHL1, oocytes expressing the T28A mutant exhibited normal high- and low-affinity nitrate transport activities (Figure 4A and B; Table I), whereas those expressing the T101A mutant had 9 or 70% of the wild-type CHL1 activity when measured using 150  $\mu$ M or 10 mM nitrate, respectively (Figure 4C and D), showing that high-affinity uptake activity was greatly reduced. Uptake measured using 10 mM nitrate includes contributions from both the low- and high-affinity components; when the contribution of the high-affinity component ( $V_{\text{max}}$  of the high-affinity component) was subtracted, the T101A mutant was found to have 138% of the low-affinity uptake of wild-type CHL1 (Figure 4D). The  $V_{\text{max}}$  values of the high-affinity component were calculated assuming that the  $K_m$  in the high-affinity phase was 50  $\mu$ M. Similar results were obtained using another two batches of oocytes isolated from another two toads (Table II). These data suggest that the inability to phosphorylate T101 converts CHL1 into an exclusively low-affinity nitrate transporter.

To mimic the phosphorylated structure, the threonine residue at 101 was changed to aspartic acid to create the mutant T101D. The uptake activity of T101D-injected oocytes measured using 150  $\mu$ M or 10 mM nitrate was 109 or 50% of that in wild-type injected oocytes, respectively (Figure 4E and F); when the contribution of the high-affinity component to the activity measured at 10 mM nitrate was taken into account, the low-affinity uptake of the T101D mutant was close to 0% compared with wild-type CHL1 (Figure 4F; Table II). Thus, by mimicking a constitutively phosphorylated state, the T101D mutation acted as a pure high-affinity nitrate transporter.

**Table II.** Nitrate uptake activities of *Xenopus* oocytes injected with mutated *CHL1*

T28A	Percentage of wild-type <i>CHL1</i> activity measured at		Percentage of wild-type <i>CHL1</i> activity calculated	
	150 $\mu$ M	10 mM	High-affinity component	Low-affinity component
Exp. 1	77	82	77	93
Exp. 2	91	92	91	92
Exp. 3	54	39	54	30

T101A	Percentage of wild-type <i>CHL1</i> activity measured at		Percentage of wild-type <i>CHL1</i> activity calculated	
	150 $\mu$ M	10 mM	High-affinity component	Low-affinity component
Exp. 1	9	70	9	138
Exp. 2	0	67	0	182
Exp. 3	0	76	0	88

T101D	Percentage of wild-type <i>CHL1</i> activity measured at		Percentage of wild-type <i>CHL1</i> activity calculated	
	150 $\mu$ M	10 mM	High-affinity component	Low-affinity component
Exp. 1	109	50	109	0
Exp. 2	114	58	114	0
Exp. 3	131	51	131	17

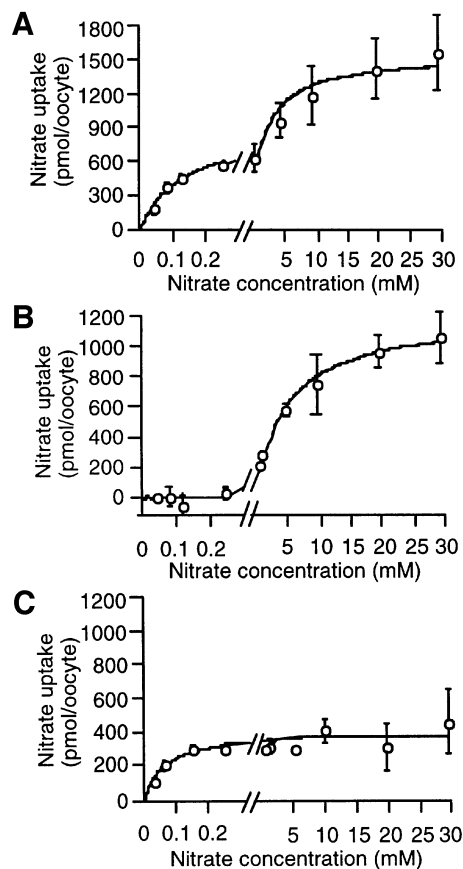
The low-affinity nitrate uptake activity was calculated by subtracting the contribution of the high-affinity component to uptake at 10 mM nitrate.

### Oocytes expressing T101A or T101D exhibit a monophasic nitrate uptake pattern

Kinetic studies were performed by measuring the uptake activities of oocytes expressing *CHL1*, *T101A* or *T101D* at nitrate concentrations ranging from 40  $\mu$ M (high-affinity range) to 30 mM (low-affinity range). As shown in Figure 5A and in our previous study (Liu *et al.*, 1999), wild-type *CHL1* exhibited a biphasic pattern, with a  $K_m$  of  $125 \pm 35 \mu$ M for the high-affinity phase and  $2.2 \pm 0.7$  mM for the low-affinity phase. In contrast, nitrate uptake by both the T101A and T101D mutants was monophasic, high-affinity activity being absent in oocytes expressing the T101A mutant (Figure 5B) and low-affinity activity being absent in oocytes expressing the T101D mutant (Figure 5C). The  $K_m$  for T101A-injected oocytes was  $\sim 5.1 \pm 1.1$  mM and that for T101D-injected oocytes was  $\sim 51 \pm 19 \mu$ M.

### Rescue of the high- but not the low-affinity uptake defect in *chl1* plants expressing the *CHL1* T101A mutant

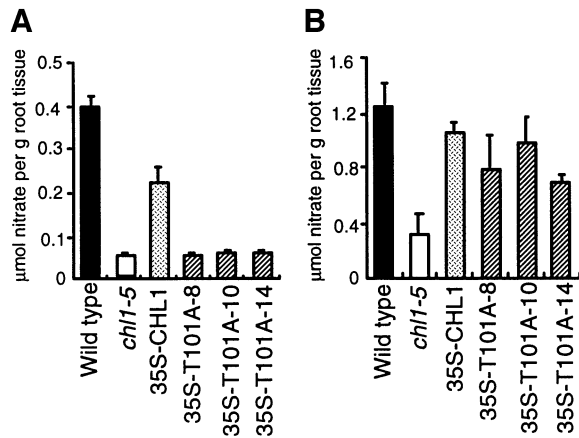
The studies described above were performed using a *Xenopus* oocyte expression system. To determine whether the phosphorylation regulatory mechanism of *CHL1* was also effective in plants, *CHL1* cDNA containing the T101A mutation was fused to the cauliflower mosaic virus 35S promoter and introduced into the *chl1* deletion mutant, *chl1-5* (Tsay *et al.*, 1993). This mutant is defective in both high- and low-affinity nitrate uptake, and transgenic *chl1-5* plants expressing wild-type *CHL1* cDNA driven by the 35S promoter can rescue the defect in both



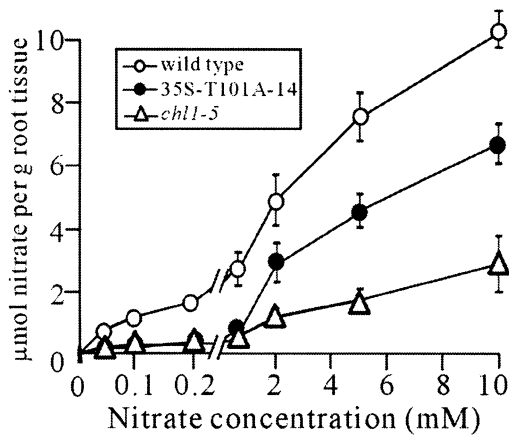
**Fig. 5.** Kinetic analysis of nitrate uptake in oocytes injected with cRNA encoding wild-type *CHL1* (A), T101A (B) or T101D (C). Uptake was determined by incubating oocytes for 1.5 h at pH 5.5 with nitrate concentrations ranging from 40  $\mu$ M to 30 mM; the part of the scale between 0 and 0.3 mM has been enlarged. Each data point is the mean of the results for three duplicate experiments, each consisting of five oocytes.

activities (Figure 4B and C; Huang *et al.*, 1996; Liu *et al.*, 1999). Consistent with the results obtained using the hetero-expressing oocyte system, three independent transgenic *chl1-5* plants expressing *T101A* failed to show recovery of the defect in high-affinity activity (Figure 6A), but did show recovery of the defect in low-affinity activity (Figure 6B).

To confirm that the T101A mutant functions as a low-affinity nitrate transporter, but not as a high-affinity transporter, a kinetic analysis of nitrate uptake activity was performed on transgenic line 35S-T101A-14, nitrate uptake activity being measured at seven different nitrate concentrations ranging from 40  $\mu$ M to 10 mM to cover both the high- and low-affinity phases of uptake. As shown in Figure 7, in wild-type plants, nitrate uptake was concentration-dependent and showed a biphasic pattern. Consistent with previous data (Wang *et al.*, 1998; Liu *et al.*, 1999), the deletion mutant, *chl1-5*, showed reduced high- and low-affinity phase uptake activity. Expression of T101A in the *chl1-5* mutant (35S-T101A-14) resulted in recovery of the low-affinity, but not the high-affinity, uptake activity. The  $K_m$  values for the low-affinity phase in the wild-type and 35S-T101A-14 plants were, respectively,  $3.7 \pm 0.6$  and  $5.2 \pm 1.7$  mM, while the corres-



**Fig. 6.** Rescue of the low-affinity, but not the high-affinity, uptake defect in *chll-5* plants expressing T101A. (A) High-affinity nitrate uptake activity of wild-type plants, *chll-5* plants or transgenic *chll-5* plants containing 35S-CHL1 or 35S-T101A; 35S-T101A-8, 35S-T101A-10 and 35S-T101A-14 are three independent transgenic T101A-expressing *chll-5* plants. Uptake activities were measured by incubating 8-day-old ammonium-grown plants in 250  $\mu\text{M}$   $\text{KNO}_3$  for 1 h. (B) Low-affinity nitrate uptake activity of the same batches of plants measured using 5 mM  $\text{KNO}_3$ .

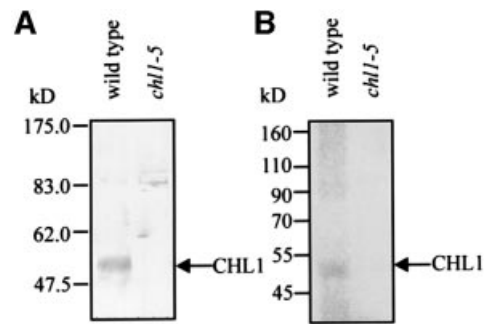


**Fig. 7.** Kinetic analysis of nitrate uptake in transgenic *chll-5* plants expressing 35S-T101A. Uptake activities of wild-type plants (white circles), the *chll-5* deletion mutant (white triangles) and the transgenic plant 35S-T101A-14 (*chll-5* expressing T101A; black circles), measured by incubating 8-day-old ammonium-grown plants in 0.04, 0.1, 0.2, 0.65, 2, 5 or 10 mM  $\text{KNO}_3$  for 40 min. The scale between 0 and 0.2 mM is enlarged. Each data point is the mean of three duplicate experiments.

ponding  $V_{\text{max}}$  values were  $13.7 \pm 0.9$  and  $10.1 \pm 1.5$   $\mu\text{mol/g}$  root tissue; in contrast, the high-affinity phase activity in the 35S-T101A-14 plant was as low as that in the *chll-5* mutant. These results show that, in plants, T101A functions exclusively as a low-affinity transporter and that the T101 phosphorylation site is required for CHL1 to function as a dual-affinity transporter.

#### CHL1 phosphorylation in plants is regulated by the nitrogen concentration

To directly demonstrate that CHL1 protein can be phosphorylated *in vivo*, metabolic labeling with

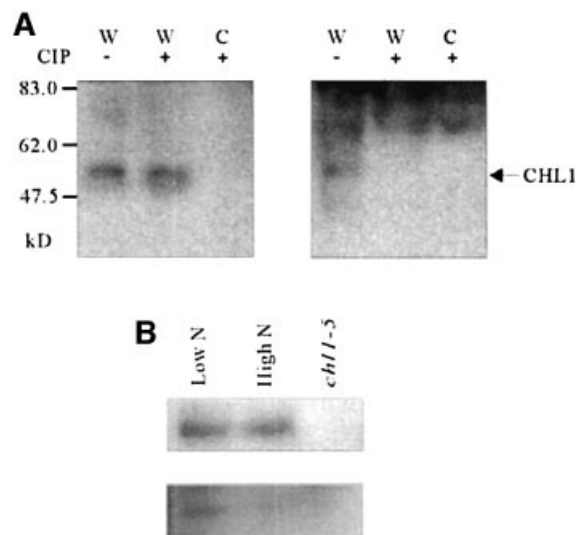


**Fig. 8.** Immunoprecipitation of  $^{32}\text{P}$ -labeled CHL1 synthesized *in vivo* in plants. (A) Immunoblot analysis of plant membrane proteins probed with anti-CHL1 antibody. Membrane proteins were isolated from root tissue of 10-day-old wild-type or *chll-5* mutant plants and 20  $\mu\text{g}$  of proteins loaded in each lane. (B) Autoradiogram of  $^{32}\text{P}$ -labeled plant membrane proteins immunoprecipitated with anti-CHL1 antibodies. Ten-day-old wild-type and *chll-5* plants were incubated with [ $^{32}\text{P}$ ]orthophosphate, then membrane proteins were extracted from the root tissue, immunoprecipitated using anti-CHL1 antibodies, and the precipitated proteins separated by SDS-PAGE. Molecular mass markers are indicated on the left. The arrows indicate CHL1.

[ $^{32}\text{P}$ ]orthophosphate was performed, followed by immunoprecipitation. Immunoblot analysis using anti-CHL1 antibodies raised against the central hydrophobic loop of CHL1 showed that a single protein with a predicted molecular mass of  $\sim 50$  kDa on the SDS gel was detected in wild-type plants, but not in the *chll-5* mutant, showing that the protein detected was the endogenous CHL1 protein and confirming the specificity of the antiserum (Figure 8A). In Figure 8B, autoradiography of  $^{32}\text{P}$ -labeled membrane proteins immunoprecipitated with anti-CHL1 antibodies revealed a protein with the same mobility as the CHL1 protein detected in immunoblots, indicating that CHL1 is phosphorylated in plants.

To detect the status of CHL1 phosphorylation at different nitrogen concentrations, anti-P-T101 antibodies were raised against phosphorylated synthetic peptide IADTFLGRYLT<sup>[P]</sup>IAIF, corresponding to residues 91–105 of CHL1, and pre-cleaned by affinity chromatography on the non-phosphorylated peptide. As shown in the right panel of Figure 9A, the pre-cleaned anti-P-T101 antibodies also detected an  $\sim 50$  kDa protein in wild-type plants, but not in the *chll-5* mutant. The specificity of the anti-P-T101 antibodies was confirmed by calf alkaline intestinal phosphatase (CIP) treatment of the membrane proteins, which resulted in elimination of the anti-P-T101 antibody signal (Figure 9A, right panel, lane 2), but not the anti-CHL1 antibody signal (left panel, lane 2), confirming that the anti-P-T101 antibodies recognize phosphorylated CHL1, but not dephosphorylated CHL1.

To determine whether CHL1 phosphorylation was regulated by the nitrogen status, 10-day-old plants were shifted for 2.5 h to low-nitrogen medium (150  $\mu\text{M}$   $\text{NH}_4\text{NO}_3$ ) or high-nitrogen medium (12.5 mM  $\text{NH}_4\text{NO}_3$ ), then membrane proteins were isolated and subjected to immunoblot analysis using anti-CHL1 antibodies (Figure 9B, top panel) or anti-P-T101 antibodies (Figure 9B, bottom panel). As shown, similar amounts of CHL1 were detected in plants grown under these two conditions, but the phosphorylation levels were different, CHL1 from plants grown under low-nitrogen conditions



**Fig. 9.** *In vivo* CHL1 phosphorylation status under different nitrogen conditions. (A) Specificity of anti-P-T101 antibodies for phosphorylated CHL1 protein. Membrane proteins were isolated from the root tissue of wild-type (W) or *chll-5* (C) plants and treated with (+) or without (–) CIP for 60 min, then 20  $\mu$ g of protein was loaded in each lane; after electrophoresis and transfer, the blots were probed with anti-CHL1 antibodies (left) or anti-P-T101 antibodies (right). (B) Regulation of CHL1 phosphorylation by nitrogen conditions. Ten-day-old wild-type plants were shifted to 150  $\mu$ M  $\text{NH}_4\text{NO}_3$  (Low N) or 12.5 mM  $\text{NH}_4\text{NO}_3$  (High N) medium for 2.5 h, then membrane proteins were isolated. The upper panel shows western blots probed with anti-CHL1 antibodies and the lower panel those probed with anti-P-T101 antibodies. Membrane protein from the *chll-5* mutant was loaded in the right-hand lane as a negative control.

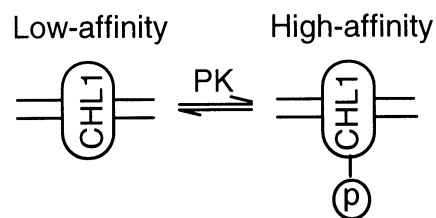
being highly phosphorylated, while that from plants grown under high-nitrogen conditions was much less phosphorylated. This result shows that a low nitrogen concentration in the medium stimulates CHL1 phosphorylation in plants.

## Discussion

### Regulation of membrane transport by phosphorylation

The transport of ions and substrates across cell membranes by pumps, channels and transporters is critical to the living cell. The activities of certain pumps, channels and transporters are known to be regulated by protein phosphorylation. In the case of pumps, phosphorylation of a threonine residue in plant  $\text{H}^+$ -ATPase creates a binding site for regulatory 14-3-3 protein (Fuglsang *et al.*, 1999; Sennelid *et al.*, 1999), while channel activities are often regulated through such processes as phosphorylation modulation of voltage sensitivity (Esguerra *et al.*, 1994; Kwak *et al.*, 1999), open probability (Wang *et al.*, 1996; Beguin *et al.*, 1999), burst duration (Beguin *et al.*, 1999) and channel complex assembly (Schiff *et al.*, 2000).

In contrast to pumps and channels, except for its effect on the trafficking of transporters to the cell surface, little is known about the detailed mechanism by which phosphorylation affects transporter activity (Blakely and Bauman, 2000). In this study, we provide both *in vitro* and *in vivo* evidence that the two action modes of the dual-affinity nitrate transporter, CHL1, are switched by phosphorylation and dephosphorylation at threonine 101. Namely, CHL1 functions as a high-affinity nitrate trans-



**Fig. 10.** Model for phosphorylation modulation of CHL1 nitrate uptake affinity.

porter when phosphorylated, and functions as a low-affinity nitrate transporter when dephosphorylated (Figure 10). Although the only two dual-affinity transporters so far identified are both plant transporters, the regulatory mechanism discovered here might be quite general and extend beyond plants. Supporting this suggestion is the report of Mehrens *et al.* (2000), which showed that the  $\text{IC}_{50}$  values of the rat organic cation transporter, rOCT1, for transported and non-transported inhibitors decrease after stimulation of protein kinase C by *sn*-1,2-dioctanoyl glycerol.

### Corresponding phosphorylation sites in other CHL1 homologs

Not every nitrate transporter in the NRT1 family exhibits dual-affinity uptake activity. For example, AtNRT1:2, a CHL1 homolog constitutively expressed in *Arabidopsis*, is a mono-affinity (low-affinity) nitrate transporter (Huang *et al.*, 1999). Consistent with the present finding, AtNRT1:2 lacks the phosphorylation sequence motif (RXXT/S) at the site corresponding to T101 of CHL1. Of the 52 CHL1 homologs in *Arabidopsis* (The Arabidopsis Genome Initiative, 2000), 36 contain this phosphorylation consensus site, but it remains to be determined whether the uptake activity of any of these is regulated by phosphorylation.

Nitrate transporters in the NRT1 family share sequence similarity with a large family known as proton-coupled oligopeptide transporters (POT) (Paulsen and Skurray, 1994), found in animals, plants, fungi, and bacteria. The human transporters, PepT1 and PepT2, two of the best characterized POT proteins, are responsible for peptide absorption in the intestine or kidney, respectively (Fei *et al.*, 1994; Liu *et al.*, 1995; Boll *et al.*, 1996; Saito *et al.*, 1996). PepT1 and PepT2 have very similar substrate specificities, recognizing a wide variety of di- and tripeptides, but differ in their affinities for peptides, PepT1 being a low-affinity transporter and PepT2 a high-affinity transporter (Leibach and Ganapathy, 1996). In contrast to the *Arabidopsis* CHL1 homologs, all of the POT transporters so far identified in animals and bacteria lack the phosphorylation consensus motif (RXXT/S) at the site corresponding to T101 of CHL1 (K.-H.Liu and Y.-F.Tsay, unpublished observation). This may indicate that, despite significant homology in the protein sequences, high-affinity nitrate uptake and high-affinity peptide uptake by the POT family have evolved by different mechanisms.

### Physiological role of the phosphorylation switch mechanism

In higher plants, in addition to CHL1 (AtNRT1), the NRT2 protein family also mediates high-affinity nitrate uptake

(Williams and Miller, 2001), raising the question of why CHL1 should have evolved dual-uptake activity, given the presence of NRT2. The answer may lie in the regulatory mechanism uncovered in this study. Once seeded, plants cannot move and therefore, in order to survive and sustain growth, must respond rapidly to changes in the environment. In contrast to the turning on and off of the expression of different mono-affinity transporters, which takes time, the phosphorylation and dephosphorylation of a protein already present in the membrane is a much more efficient way of adjusting high- and low-affinity uptake capacity in response to environmental changes. One particular example is when nutrient levels in the soil are low and diffusion limits nutrient movement to the root surface, resulting in a nutrient-depleted zone adjacent to the root surface (Mengel and Kirkby, 1987). In this situation, nutrient absorption relies on the high-affinity uptake system. In order to find a new nutrient source, the roots grow out into fresh soil and, when nutrients are found, the plant can then take advantage of the higher capacity of the low-affinity uptake system. Having the ability to switch between high- and low-affinity modes simply by phosphorylation/dephosphorylation, CHL1 can rapidly change its affinity as the root grows. In support of this idea are the following observations: (i) CHL1 phosphorylation was high when plants were grown under nitrogen-limited conditions, but undetectable when plants were exposed to a high nitrogen concentration (Figure 9); (ii) the *CHL1* gene is abundantly expressed in the root tip (Huang *et al.*, 1996; Guo *et al.*, 2001); (iii) CHL1 is localized in the plasma membrane (S.-C.Hou and Y.-F.Tsay, unpublished data); and (iv) the *chl1* mutant exhibits a root growth defect (Guo *et al.*, 2001). Taken together, the dynamic regulatory mechanism described in this study could be a key player in the plant's fight for limited nitrogen.

## Materials and methods

### Plasmid construction and site-directed mutagenesis

Point mutations T101A, T101D, and T28A were introduced using the recombinant PCR technique described previously (Higuchi, 1990). Wild-type CHL1 cDNA was cloned into the pGEMHE vector (Limman *et al.*, 1992) as a template with *Bam*HI, *Eco*RI and *Xho*I cloning sites on the 5' end of the CHL1 cDNA, and *Hind*III, *Xba*I, and *Eco*RI cloning sites on the 3' end. PCR amplifications were performed using VioTaq DNA polymerase (Viogene, Taiwan). T101A and T101D cDNAs were first prepared by PCR amplification using a T101A primer or T101D primer, respectively, in conjunction with a CHL1C primer, then the amplified DNA fragments were gel purified, digested with *Kpn*I and *Hind*III, and used to replace the corresponding fragment in wild-type pGEMHE-CHL1 to generate the plasmids, pGEMHE-T101A and pGEMHE-T101D.

To generate T28A cDNA, two primers, the T28A forward primer and T28A reverse primer, were synthesized. In these, the CHL1 ATCGCT close to the sequence coding for threonine 28 was replaced with ATCGAT to create a *Clal* cloning site without changing the amino acid sequence (both CGC and CGA code for arginine). In addition to this newly generated *Clal* site, wild-type CHL1 cDNA has an endogenous *Clal* site located 600 bp upstream of its stop codon. Two steps were needed to generate the plasmid pGEMHE-T28A. In the first, pGEMHE-CHL1 was digested with *Bam*HI and *Clal*, and the 3.6 kb fragment containing the pGEMHE vector and the 0.6 kb 3' portion of CHL1 cDNA gel were purified. The 109 bp DNA fragment amplified with the CHL1N primer and T28A reverse primer was digested with *Bam*HI and *Clal*, and subcloned into the 3.6 kb fragment described above to generate plasmid pGEMHE-CHL1-109. In the second step, a 1.1 kb DNA fragment amplified with the T28A forward primer and CHL1C primer was produced by *Clal* digestion and cloned into the *Clal* site of pGEMHE-CHL1-109 to generate plasmid pGEMHE-T28A.

The sequences of the primers used were: T101A primer: 5'-GGGCAG-TACCTAGCGATTGC-3' (sense, *Kpn*I), T101D primer: 5'-GGGCAG-GTACCTAGACATTGC-3' (sense, *Kpn*I), CHL1C primer: 5'-CCC-AAGCTTCATGATCAATGACCCATTGG-3' (antisense, *Hind*III), CHL1N primer: 5'-CCCGATCCGATCATTAAATCCATCTTCAAG-3' (sense, *Bam*HI), T28A reverse primer: 5'-TTGATCGATCGGCGGG-3' (antisense, *Clal*) and T28A forward primer: 5'-CCGATCGATCAA-AAGCCG-3' (sense, *Clal*). The substitution sites for the threonine 101 residue (ACG) and threonine 28 residue (ACC) are underlined; restriction sites are shown in bold.

### *Xenopus* oocyte nitrate uptake assay

Oocytes were isolated and injected with 50 ng of *in vitro* synthesized CHL1 cRNA, T28A cRNA, T101A cRNA, or T101D cRNA as described previously (Tsay *et al.*, 1993). In PKA activation experiments, oocytes were pre-incubated for 90 min in ND-96 medium (Roche and Treistman, 1998), containing 0.5 mM 8-Br-cAMP (a cAMP analog), 50  $\mu$ M forskolin (an activator of adenylate cyclase) and 0.5 mM IBMX (an inhibitor of the cAMP degrading enzyme, phosphodiesterase), in order to increase the cytosolic cAMP concentration (Maurel *et al.*, 1995). In PKA inhibition experiments, oocytes were pre-incubated for 90 min in ND-96 medium containing 50  $\mu$ M H89. In nitrate uptake studies, oocytes were incubated for 90 min in medium containing the indicated nitrate concentration, and the amount of nitrate retained in the oocyte (low-affinity experiments) or depleted from the solution (high-affinity experiments) was determined by HPLC (Liu *et al.*, 1999).

### *In vitro* phosphorylation and immunoprecipitation of oocyte membranes

Anti-CHL1 antibody was raised in rabbits against a purified recombinant protein corresponding to the 94 amino acid central loop of CHL1, which shares least homology with other *Arabidopsis* CHL1 homologs. Two hundred CHL1- or water-injected oocytes were homogenized in 5 ml of 30 mM Tris-HCl pH 7.6, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA and 1 mM phenylmethylsulfonyl fluoride (PMSF), then membranes were collected as described by Maurel *et al.* (1995). For immunoblot analysis, membrane protein from 50 oocytes was loaded in each lane. For *in vitro* phosphorylation, the same amount of protein was incubated for 15 min at 30°C in 100  $\mu$ l of 500 mM HEPES pH 7.5, 10 mM MgCl<sub>2</sub>, 0.2% Triton X-100, 1 mM dithiothreitol and 25  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP (NEN Life Science Products, MA), containing 2.5 U of cAMP-dependent kinase catalytic subunit (New England Biolabs, MA), then the reaction was stopped by placing the tubes at 0°C and adding a final concentration of 7.2% TCA. The labeled membrane proteins were resuspended in buffer I (50 mM Tris-HCl pH 7.6, 200 mM NaCl, 50 mM NaF, 50 mM EDTA, 5 mM EGTA, 5 mM  $\beta$ -glycerophosphate, 1 mM PMSF) containing 1.5% SDS, then the solubilized membrane proteins were incubated overnight at 4°C with anti-CHL1 antiserum in buffer I containing 1% Triton X-100 and 0.2% SDS. Immune complexes were absorbed onto protein A-Sepharose CL-4B (Sigma), which was washed as described by Maurel *et al.* (1995), then the bound proteins were eluted at 100°C for 5 min with 50  $\mu$ l of SDS sample buffer (12% SDS, 6% mercaptoethanol, 30% glycerol, 0.05% Serva blue G, 150 mM Tris-HCl pH 7) and separated on a 10% SDS-PAGE gel.

### Phosphoamino acid analysis

Phosphoamino acid analysis of *in vitro* phosphorylated CHL1 protein was performed as described by Xu *et al.* (1996).

### *Arabidopsis* nitrate uptake assay

The T101A CHL1 mutant cDNA was fused to the cauliflower mosaic virus 35S promoter, then cloned into the binary vector, pBIN19, to produce plasmid p35S-T101A, which was introduced into the CHL1 deletion mutant, *chl1-5*, using the *in planta* vacuum infiltration procedure (Bechtold *et al.*, 1993). Three independent transgenic plants, 35S-T101A-8, 35S-T101A-10 and 35S-T101A-14, were isolated and their inserts confirmed by kanamycin resistance and genomic DNA gel blot analysis; 35S-T101A-8 and 35S-T101A-14 contained a single copy of the insert, whereas 35S-T101A-10 contained two copies. Plants were grown in 50 ml flasks containing 10 ml of liquid medium with 12.5 mM (NH<sub>4</sub>)<sub>2</sub> succinate as the sole nitrogen source at pH 6.5. Nitrate uptake activities of 8-day-old plants were determined over a 30 min period in 4 ml of medium containing 250  $\mu$ M KNO<sub>3</sub> for high-affinity measurements and in 2.5 ml of medium containing 5 mM KNO<sub>3</sub> for low-affinity measurements. For kinetic analysis, nitrate uptake activities of 8-day-old plants were determined over a 40 min period in 2–4 ml of medium containing the indicated KNO<sub>3</sub> concentration. The kinetic parameters, presented as

estimated  $K_m$  or  $V_{max} \pm$  error, were calculated by fitting the data to the Michaelis–Menten equation using a non-linear least-squares method in the program Origin 5.0 (Microcal Software Inc., Northampton, MA).

### **In vivo phosphorylation study in Arabidopsis**

Unless specified, the plants used for immunoblot analysis and *in vivo* labeling were grown hydroponically for 10 days on mesh in Magenta vessels (Sigma) containing 50 ml of liquid medium with 12.5 mM  $\text{NH}_4\text{NO}_3$  as the sole nitrogen source at pH 5.5.

For *in vivo* labeling experiments, the seedlings were washed three times with phosphate-free medium containing 12.5 mM  $\text{NH}_4\text{NO}_3$ , and incubated for 2 h in phosphate-free medium, then for 3 h in phosphate-free medium containing 5 mCi [ $^{32}\text{P}$ ]orthophosphate. The roots were homogenized in homogenization buffer [15 mM Tris–HCl pH 7.8, 250 mM sucrose, 1 mM EDTA, 2 mM DTT, 1 mM PMSF, 0.6% (w/v) polyvinylpyrrolidone and 50 mM  $\beta$ -glycerophosphate] and membranes collected as described by Guo *et al.* (2001). They were then resuspended in homogenization buffer and diluted 100-fold in 20 mM  $\text{Na}_2\text{HPO}_4 \cdot \text{NaH}_2\text{PO}_4$  buffer pH 7.0, containing 250 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF and 50 mM  $\beta$ -glycerophosphate. The diluted samples were pre-cleared by incubation for 15 min with protein A–Sepharose CL-4B beads and removal of the protein A–Sepharose CL-4B beads by centrifugation, then anti-CHL1 antibodies pre-incubated for 30 min at 4°C with membrane proteins from *chl1-5* to remove non-specific binding were added and the mixture incubated for 3 h at 4°C. The immune complexes formed were absorbed onto protein A–Sepharose CL-4B (Sigma), which was then washed four times with wash buffer (20 mM  $\text{Na}_2\text{HPO}_4 \cdot \text{NaH}_2\text{PO}_4$  buffer pH 7.4, 150 mM NaCl, 1% Triton X-100) and twice with wash buffer without 1% Triton X-100, as described by Adan *et al.* (2000) and eluted for 5 min at 100°C with 50  $\mu\text{l}$  of SDS sample buffer. The eluted proteins were then separated on an 8% SDS–PAGE gel.

To determine the phosphorylation status of CHL1, a rabbit antiserum against CHL1 phosphorylated at T101 (anti-P-T101 antibodies) was raised by Pro-e Biotech (Taiwan) using the synthetic peptide, IADTFLGRYLT<sup>101</sup>IAIF (residues 91–105 of T101-phosphorylated CHL1) as antigen. The antiserum was pre-cleaned by affinity chromatography using the corresponding non-phosphorylated CHL1 peptide (IADTFLGRYLTIAIF) coupled to agarose (Pro-e Biotech). To check the specificity of the antibodies for T101-phosphorylated CHL1, membrane protein extracts from wild-type plants and the *chl1-5* mutant were prepared in the absence of 50 mM  $\beta$ -glycerophosphate, then diluted ~1:5 in 1 $\times$  reaction buffer 3 (New England BioLabs), treated for 60 min at 37°C with buffer or CIP (Promega; 20 U of CIP and 40  $\mu\text{g}$  of membrane protein in a volume of 80  $\mu\text{l}$ ), then tested for antibody binding on western blots. To test the phosphorylation status of CHL1, seedlings were washed three times and shifted to 150  $\mu\text{M}$   $\text{NH}_4\text{NO}_3$  or 12.5 mM  $\text{NH}_4\text{NO}_3$  medium for 2.5 h, then root tissue membrane proteins were extracted (Guo *et al.*, 2001) and 20  $\mu\text{g}$  of the sample separated on SDS–PAGE gels, transferred to PVDF membranes and tested for binding of anti-CHL1 and anti-P-T101 antibodies.

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