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Phosphorylation of *Arabidopsis* NRT1.1 regulates plant stomatal aperture and drought resistance in low nitrate condition

Yuchen Kou[1†], Bodan Su[3†], Shunyao Yang[1], Wei Gong[1], Xi Zhang[1*] and Xiaoyi Shan[2*]

Abstract

Background   NITRATE TRANSPORTER 1.1 (NRT1.1) functions as a dual affinity nitrate transceptor regulated by phosphorylation at threonine residue 101 (T101). Previous studies have suggested that NRT1.1 is involved in stomatal opening and contributes to drought susceptibility. However, the precise mechanism of how the phosphorylation status of NRT1.1 affects stomatal movement and drought tolerance remains unclear.

Results   In this study, we observed that seedlings expressing the phosphorylated form of NRT1.1 (NRT1.1[T101D], T101D) exhibited increased drought tolerance compared to dephosphorylated NRT1.1 (NRT1.1[T101A], T101A) mutants under low nitrate (LN) condition, characterized by decreased stomatal aperture and water loss. Moreover, we found that the drought-induced depolarization of membrane potential was diminished in T101D mutants in comparison to T101A seedlings. Furthermore, we revealed that the reduced stomatal opening in T101D seedlings was related with depressed nitrate and potassium influx, along with the down-regulation of *NRT1.1*, *POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1*, and *ARABIDOPSIS* H$^+$ *ATPase 1* in comparison with that of T101A.

Conclusions   Our study provides several lines of evidence to demonstrate that the phosphorylation of NRT1.1 at T101 contributes to the drought tolerance under LN condition by reducing the influx of nitrate and potassium into the cytoplasm, attenuating membrane depolarization and thereby inducing stomatal closure. This finding identified a novel drought resistance mechanism enabled by post-transcriptional regulation of plasma membrane transporter.

Keywords   NRT1.1, Phosphorylation, Drought tolerance, Stomatal movement, Nitrate influx

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Background

Stomatal movement, a critical event for regulation of transpirational water loss and drought tolerance, is highly regulated by multiple signal pathways [1, 2]. The opening and closing of guard cells are mainly controlled by changes of cell volume that are closely associated with influx and efflux of different ions [1, 3–5]. The inward potassium (K^+) current in guard cells plays a key role in stomatal opening. In this process, activation of plasma membrane (PM) H^+-ATPases such as ARABIDOPSIS H^+ ATPase 1 (AHA1) generates an electrochemical gradient to turn on the hyperpolarization-gated K^+ channel 3-KETO-ACYL-COA THIOLASE 1 (KAT1). In contrast, the PM-located S-type anion channels and tonoplast-located CHLORIDE CHANNEL C (AtCLCc) mediate the chloride (Cl^-) efflux and translocation to reduce the water potential as well as facilitate stomatal closure. Moreover, nitrate (NO_3^-) directly induces the stomatal opening under light conditions, whereas high permeability for nitrate over chloride, with a ratio $[P(NO_3^-)/P(Cl^-)]$ of ~ 21, contributes to fast stomatal closure under drought stress [6, 7].

NITRATE TRANSPORTER 1.1 (NRT1.1), a PM-located nitrate transceptor, fulfills its dual role by switching the phosphorylation form of threonine-101 (T101) [8, 9]. Under low nitrate (LN) condition, phosphorylated NRT1.1 serves as a high-affinity nitrate transporter and prevents higher primary nitrate response (PNR) [8, 9]. In response to high nitrate (HN) treatment, unphosphorylated NRT1.1 serves as a low-affinity nitrate transporter and generates a high PNR [8, 9]. NRT1.1 has been identified as a key regulator involved in root architecture including primary root and lateral root (LR) development [10–16]. NRT1.1 also functions in stomatal movement and contributes to drought susceptibility [6, 17]. In the presence of nitrate, *NRT1.1* mutants (*chl1-4* and *chl1-5*) showed reduced nitrate accumulation and nitrate-induced depolarization of guard cells, thereby inhibiting stomatal opening and enhancing drought tolerance [6, 18]. Recently, genetic and physiological evidence revealed that the phosphorylation of T101 contributes to the modulation of NRT1.1-mediated LR development [10, 16], whereas no clue on how phosphorylation forms of NRT1.1 regulate the stomatal opening and drought tolerance of plants.

In this study, we provided several lines of evidence to prove that the phosphorylation of NRT1.1 at T101 could enhance plant drought tolerance under LN condition. The NRT1.1^{T101D} (T101D) mutants showed compromised expression of *NRT1.1*, *KAT1*, *AHA1* and reduced influx of NO_3^- and K^+, which reduces PM depolarization and promotes stomatal closure, thereby resulting in lesser water loss and improved drought resistance.

Collectively, our data reveal the role of NRT1.1 phosphorylation in regulating drought stress response under LN condition, providing new insights into how plants coordinately respond to nutrition deficiency and drought stress by membrane transport and signaling.

Methods

Plant materials

The *Arabidopsis thaliana* seedlings used in this study were Columbia (Col-0, WT) and NRT1.1 deletion mutant *chl1-5*. Transgenic plants were *pNRT1.1::NRT1.1^{T101D}/chl1-5* (hereafter abbreviated T101D) and *pNRT1.1::NRT1.1^{T101A}/chl1-5* (hereafter abbreviated T101A), which replace the phosphorylation site threonine (Thr, T) of NRT1.1 with the charged aspartic acid (Asp, D) and uncharged alanine (Ala, A) respectively, to achieve the phosphomimetic T101D mutant and non-phosphorylatable T101A mutant. The *chl1-5*, T101A, and T101D seeds were kindly provided by Dr. Yi-Fang Tasy [8].

Plant growth conditions

Arabidopsis thaliana seeds were routinely surface-sterilized for 2 min in 85% EtOH:H_2O_2 = 4:1 (v/v), plated on 1/2 MS medium or basic medium containing 12.5 mM $(NH_4)_2$ $C_4H_4O_4$, 0.5 mM $CaSO_4$, 0.5 mM $MgCl_2$, 1 mM KH_2PO_4, 2.5 mM MES (pH 6.5), 50 μM NaFeEDTA, 50 μM H_3BO_3, 12 μM $MnCl_2$, 1 μM $CuCl_2$, 1 μM $ZnCl_2$, 0.03 μM NH_4MoO_4 with different concentrations of KNO_3, vernalized at 4 °C for 2 d, and transferred to a light incubator for 7 days at a photo-period of 16 h (light)/8 h (dark) (100 μmol m^{-2} s^{-1}) at 23 °C/20 °C, then moved into the soil for growth, watering regularly.

Drought tolerance test and survival rate measurement

Drought tolerance test and survival rate measurement were carried out with appropriate improvements as previously described [6]. For drought tolerance test, 15-day-old seedlings grown on 1/2 MS medium were shifted to pots filled with vermiculites and watered with basic medium containing 0.2 mM or 30 mM NO_3^- respectively. Following that, seedlings were transferred to an incubator and then drought stressed by terminating irrigation. During dehydration treatment, the seedlings are divided into two categories, one is living seedlings, another is withered seedlings with withered and yellow leaves. The survival rate was calculated as the ratio of the number of living seedlings to the total number of seedlings.

Water loss assay

In the water loss assay, 15-day-old seedlings cultivated on 1/2 MS medium were shifted to basic medium containing 0.2 mM or 10 mM NO_3^- respectively and allowed to grow for 6 days. Subsequently, the weight of the seedlings

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was recorded every 15 min for a duration of 180 min post-root detachment. Similarly, for detached leaves, the weight was monitored every 15 min for up to 240 min. Finally, the water loss rate was presented as the percentage of water loss relative to the initial fresh weight (FW) at each time point.

Stomatal aperture measurement

For stomatal aperture measurement, 7-day-old seedlings grown on 1/2 MS medium were immersed in the nitrate-free solution for nitrogen starvation. After dark overnight, seedlings were transferred into basic medium solution containing 0.2 mM or 10 mM NO_3^- respectively to induce stomatal opening under the light condition for 3 h. Seedlings kept in the dark were set as negative controls. The stomatal was captured by a Leica TCS SP8 Confocal Microscope (Leica, Germany) fitted with a 63× oil-immersion objective. Stomatal aperture was quantified using ImageJ (1.51w) software.

Imaging membrane potential changes in intact guard cells

Seven-day-old seedlings grown on 1/2 MS medium were first immersed into the loading buffer (5 mM Mes-KOH, 0.25 mM KCl, and 1 mM $CaCl_2$, adjusted pH to 5.7 with by KOH), and then incubated in loading buffer containing 1 μM B-438 dye [bis-(1,3-dibutylbarbituric acid) trimethine oxonol (Sigma-Aldrich, Darmstadt, Germany)] for 10 min. The images were collected every 5 s after adding KNO_3 or KCl solutions up to 140 s. The experiment was performed on an inverted OLYMPUS FV1200 microscope through a water immersion objective (63×, numerical aperture = 1.2). An argon laser (488 nm line) was used for excitation and detected from a 510 to 555-nm bandpass filter (green). Analysis of fluorescence intensity was carried out by ImageJ software.

Nitrate uptake assay in seedlings

Seedlings were grown in nutrition soil for 4 weeks and then placed in the dark overnight. Detached leaves were floated in basic medium solution containing 0.2 mM or 10 mM NO_3^- with light for 2 h. Epidermal strips from detached leaves were balanced for 15 min in measuring solution (0.2 mM $CaCl_2$, 0.1 mM KCl, 0.2 mM KNO_3 and 0.5 g L^{-1} MES, adjusted pH to 5.8 with 1 M NaOH). The net NO_3^- and K^+ flux of each leaf was measured by Non-invasive Micro-test Technology (NMT) technique with BIO-003A system (Amherst MA, YoungerUSA, LLC, youngerusa.com) in Xuyue Beijing Sci. & Tech. Co. Ltd. (xuyue.net) at room temperature. The microelectrode was vibrated in the measuring solution between 5 and 35 μm from the guard cell surface along an axis perpendicular to the guard cell. Discarded the first 5 min

of fluctuation data, and the following stable data were recorded up to 10 min. The data were analyzed by the Mage Flux program (http://www.xuyue.net/mageflux).

Quantitative real-time PCR

Ten-day-old seedlings grown on basic medium containing 0.2 mM NO_3^-, and leaves were harvested for total RNA extraction and quantitative real-time PCR (qRT-PCR) assays. Total RNA was extracted from homogenized leaves by using RNA prep Pure Plant Kit (TIANGEN BIOTECH (BEIJING) CO., LTD.). The cDNA was synthesized from the RNA with oligo(dT)$_{18}$ as a primer and Moloney murine leukaemia virus (M-MLV) reverse transcriptase using PrimeScript™ 1st Strand Synthesis Kit (Takara Biomedical Technology (Beijing) Co., Ltd.). Gene expression was determined by SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio Inc. Japan) and gene-specific primers (listed in Tables S1) on a BIO-RAD CFX Connect™ real-time PCR system (BIO-RAD, Hercules, California, American). The quantification method ($2^{-\Delta\Delta CT}$(DDCT)) was used to evaluate quantitative variation.

Results

T101D mutants are more drought tolerant than T101A mutants under LN condition

To investigate whether the phosphorylation state of NRT1.1^{T101} affects plant drought tolerance, we measured the survival rate (SR) of WT (wild-type, Col-0), *chl1-5* (total deletion of NRT1.1), *pNRT1.1::NRT1.1^{T101A}/chl1-5* (a non-phosphorylatable T101A mutant, hereafter abbreviated T101A), and *pNRT1.1::NRT1.1^{T101D}/chl1-5* (a phosphomimetic T101D mutant, hereafter abbreviated as T101D) (Fig. S1) by dehydration treatment for the 15-day-old seedlings [8]. When grown under LN condition (0.2 mM NO_3^-), significantly higher SR was observed for T101D seedlings relative to WT plants while the SR of T101A plants was obviously lower than that of WT plants (for example, 46.7% of T101D, 13.3% of T101A and 33.3% of WT at 30 days after dehydration treatment) (Fig. 1a, c; Fig. S2a). In addition, *chl1-5* mutant exhibited an enhanced drought-resistant phenotype in comparison to T101D (for example, 60.0% SR at 30 days after dehydration treatment)) (Fig. 1a, c; Fig. S2a). When grown under HN conditions (30 mM NO_3^-), there were more alive T101D plants than T101A and WT seedlings, which was similar to *chl1-5* mutant (for instance, 66.7% of T101D, 53.3% of T101A, 40.0% of WT and 60.0% of *chl1-5* at 30 days after dehydration treatment) (Fig. 1b, d; Fig. S2b). These data indicate that T101D, and by extension, phosphorylated WT NRT1.1 improves plant tolerance to drought stress in response to different concentrations of nitrate.

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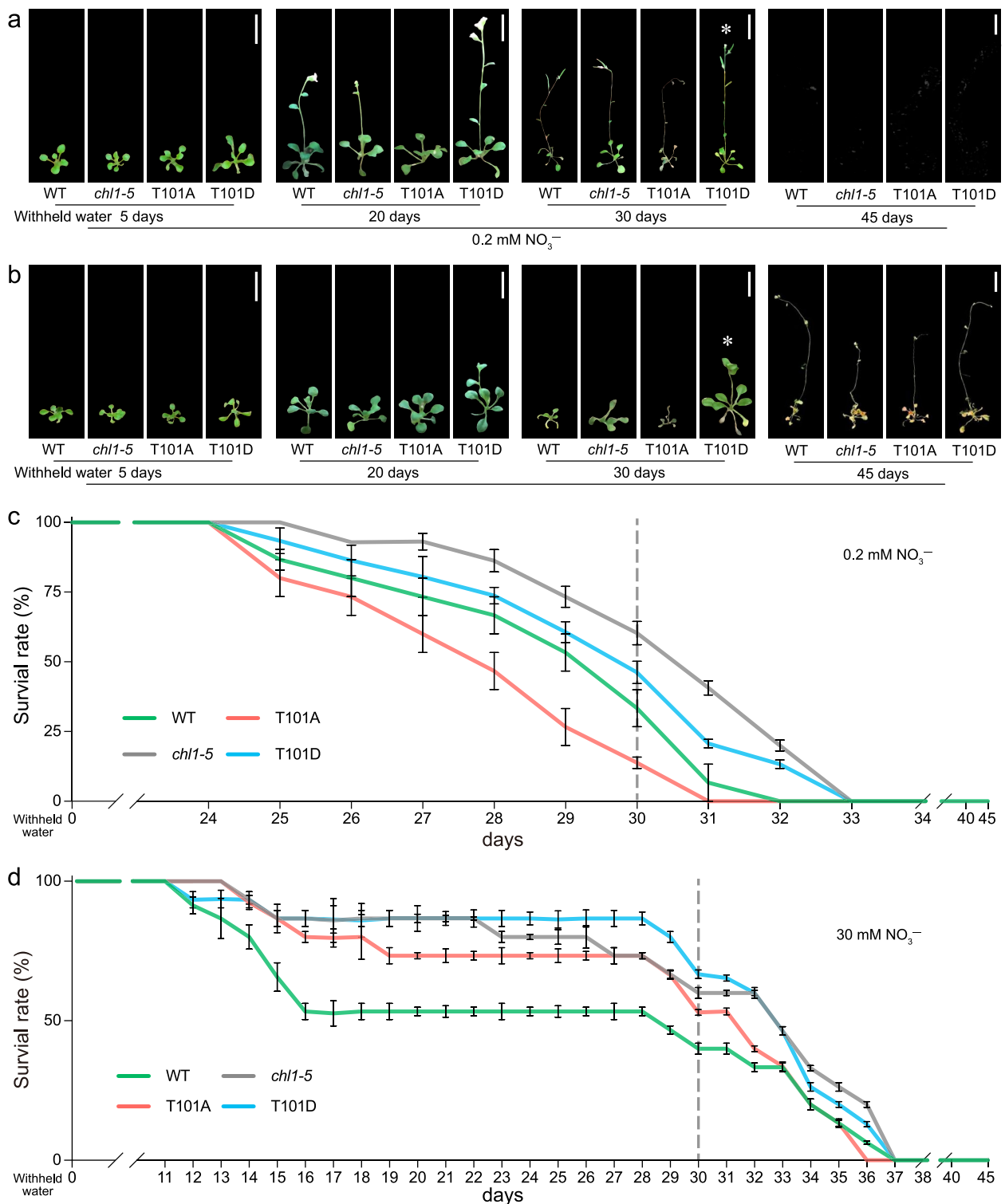
Fig. 1 T101D mutants are more drought tolerant than T101A mutants.(**a**, **b**) Drought resistance of WT, *chl1-5*, T101A and T101D plants grown under 0.2 mM (LN) (**a**) and 30 mM (HN) (**b**) nitrate-containing medium by dehydration treatment. Representative photographs were taken at 5, 20, 30 and 45 days after withholding water. Bar = 1 cm. (**c**, **d**) The survival rate of WT, *chl1-5*, T101A, and T101D plants grown under LN (**c**) and HN (**d**) conditions by dehydration treatment. Data are means ± SEM (*n* = 3). For (**a**-**d**), seedlings were grown on 1/2 MS medium for 15 days and transferred to vermiculite medium irrigated with LN or HN medium, and then subjected to drought stress by terminating irrigation. 15 seedlings for each genotype were examined for survival rate. These experiments were repeated three times

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Since T101D mutation led to increased drought tolerance, it is plausible that the phosphorylation of T101 might influence the transpiration of seedlings. Thus, the water loss assay was performed on the whole seedlings of T101A and T101D plants grown on 0.2 mM (LN) and 10 mM (HN) NO_3^--containing medium during 180 min of desiccation respectively. In response to LN treatment, the fresh weight of T101D mutants decreased more slowly than that of T101A especially in the early stage of dehydration (within 75 min) (Fig. 2a, c). However, T101D and T101A plants did not show any evident difference in water loss at most time points when exposed to HN condition (Fig. 2b, d). In addition, the parallel experiment showed that mature *chl1-5* mutants lost water faster than WT plants upon 0.2 mM and 10 mM nitrate treatment, possibly due to significant growth differences between these two lines (Fig. S3). We further compared the rate of water loss in detached leaves of WT, *chl1-5*, T101A, T101D plants grown under LN condition (0.2 mM NO_3^-). Water loss was considerably slower in T101D compared with T101A and WT leaves, with leaves of *chl1-5* plants exhibiting the lowest level of water loss over the same period (Fig. 2e, f). Taken together, these results demonstrate that the phosphorylation on NRT1.1^{T101} could prevent water loss of leaves especially under LN conditions, thereby leading to increased drought tolerance.

T101D mutants exhibited reduced stomatal aperture compared with T101A under LN condition

Stomatal aperture is in close contact with water loss and leaves transpiration [19]. Considering the strong expression of *NRT1.1* in guard cells and the significant difference of water loss between T101A and T101D detached leaves, we examined the role of NRT1.1 phosphorylation in regulating stomatal aperture on intact leaves of WT, *chl1-5*, T101A, and T101D seedlings. 7-day-old seedlings grown on 1/2 MS medium were exposed to dark for complete stomatal closure (Fig. 3a, b). When treated with 0.2 mM NO_3^- plus 3 h light induction, T101D had a more substantial effect on inhibition of stomatal opening which was comparable to *chl1-5* seedlings (Fig. 3a, b). The stomatal apertures of T101D were 32% and 41% smaller than that of WT and T101A plants, respectively (Fig. 3a, b). However, the light-induced stomatal aperture of T101D was similar to that of WT and T101A upon 10 mM NO_3^- treatment (5.0, 5.2 and 5.5 μm for WT, T101D and T101A respectively) but was much larger than *chl1-5* mutant (4.2 μm) (Fig. 3a, b). Thus, we conclude that phosphorylated NRT1.1 appears to impede light-induced stomatal opening under LN condition.

The changes in membrane potential are known to be associated with stomatal movement. In guard cells, NRT1.1 functions as an electrogenic nitrate transporter that depolarizes the PM to facilitate stomatal opening [6]. To gain insight into the role of distinct NRT1.1 phosphorylation variants in nitrate-induced PM depolarization, we examined the membrane potential in guard cells of WT, *chl1-5*, T101A, and T101D by voltage-sensitive dye bis-oxonol (B-438) in presence of 0.2 mM NO_3^-. Based on the confocal images and the quantitative analysis of WT samples, we proposed 90 s as a suitable length of time to monitor the changes of membrane potential after 0.2 mM NO_3^- stimulation (Fig. S4a, b). As a control, nitrate-free treatment failed to trigger the PM depolarization in guard cells of each genotype (Fig. 3c; Fig. S4c, d). Application of 0.2 mM NO_3^- induced a strong PM depolarization in the guard cell of WT and T101A plants as depicted by the greenish pseudo-color on the heat map, whereas a 'blue-shift' was observed in the guard cells of T101D, indicating a significant reduction of PM depolarization (Fig. 3d). In addition, no obvious fluorescence was observed in the guard cells of the *chl1-5* mutant even under the LN condition (Fig. 3d). Statistical analysis of the fluorescence signal confirmed the changes in membrane potential (Fig. 3e, f). Collectively, these data clearly suggest that nitrate-induced membrane depolarization of guard cells is defective in the phosphomimetic mutant of NRT1.1, which results in inhibition of stomatal opening.

T101D mutants showed lesser NO_3^- and K^+ influx in the guard cells than T101A under LN condition

The membrane depolarization is often induced by rapid influx/efflux of ions (such as K^+, H^+, Cl^- and NO_3^-) across the PM. To determine whether the phosphorylation form of NRT1.1 impacts the NO_3^- flux in the guard cell, we recorded the NO_3^- flow rates in guard cells of WT, *chl1-5*, T101A, and T101D seedlings by non-invasive microtest technology (NMT). Upon treatment with 0.2 mM NO_3^-, T101A showed an instantaneous NO_3^- influx in guard cells, with the mean influx of -8.91 pmol cm^{-2} s^{-1} (Fig. 4a, b). However, the NO_3^- flux occurred as a stable efflux in WT, *chl1-5* and T101D seedlings (28.9, 133.4, and 15.8 pmol cm^{-2} s^{-1}, respectively), among which the efflux in *chl1-5* mutants was much higher than that in WT and T101D seedlings (Fig. 4a, b). In the parallel NMT experiments under 10 mM NO_3^- treatment, the trend of transmembrane NO_3^- efflux in guard cells of WT, T101D and *chl1-5* was similar to that under 0.2 nM nitrate treatment (Fig. 4c, d). T101A also presented a net efflux pattern and had no significant difference compared to that in the WT and T101D guard cells (Fig. 4c, d). These results indicate that the NO_3^- flux in guard cells is substantially changed by the phosphorylation modification on T101 of NRT1.1 under LN condition.

Guard-cell K^+ current always functions in the modulation of PM potential and stoma movement [20].
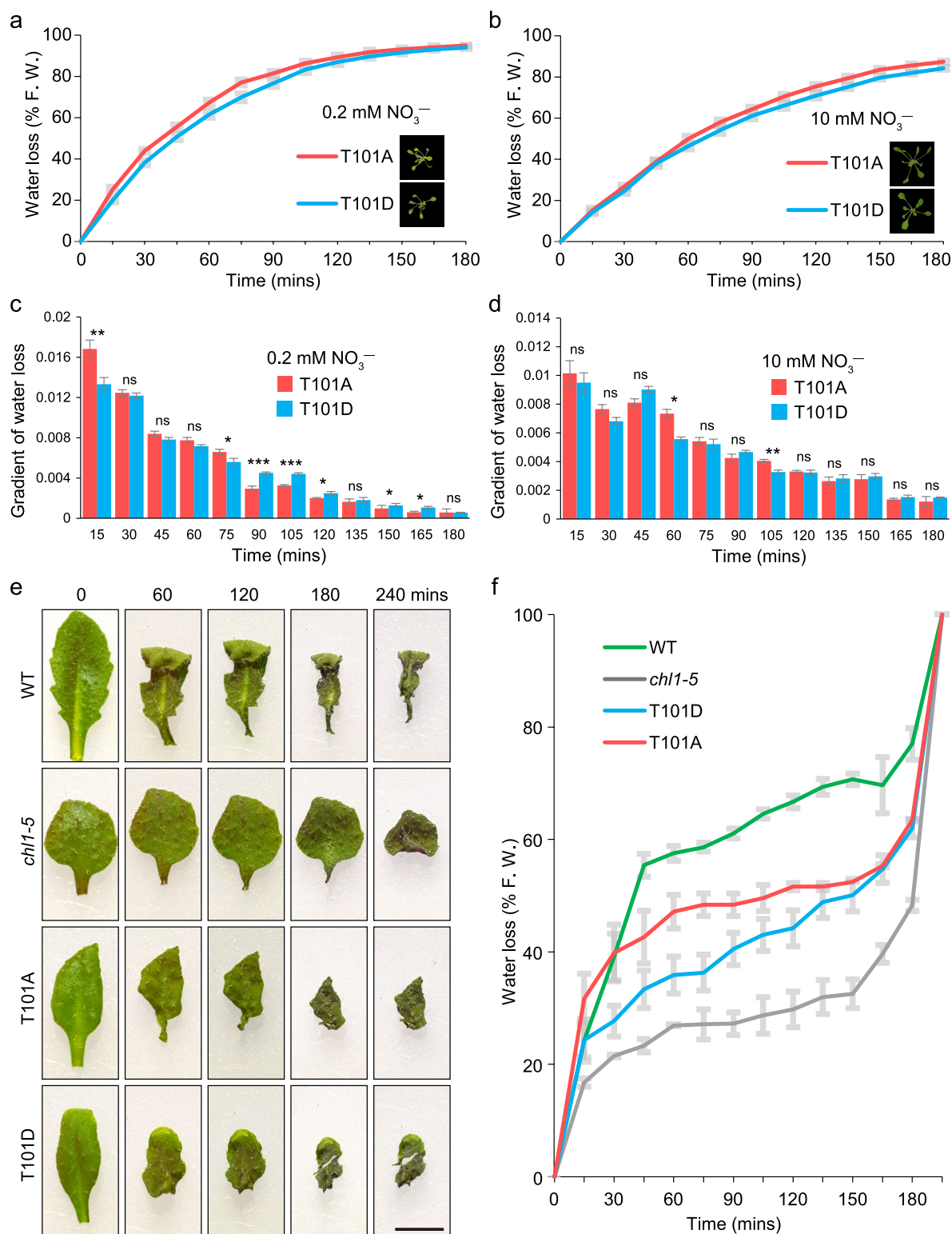
**Fig. 2** T101D mutants lost water more slowly than T101A mutants under LN condition. (**a**, **b**) Water loss of WT, *chl1-5*, T101A and T101D seedlings grown under LN (**a**) and HN (**b**) conditions at indicated time points during 180-min dehydration. (**c**, **d**) The gradient of T101A and T101D water loss calculated from a (**c**) and b (**d**), (**e**, **f**) The representative phenotypes (**e**) and water loss (**f**) of detached leaves from WT, *chl1-5*, T101A, and T101D at indicated time points during 240-min dehydration under LN condition. Bar = 1 cm. For (**a**-**f**), 15-day-old seedlings grown on 1/2 MS medium were shifted into basic medium containing 0.2 mM (LN) or 10 mM (HN) NO_3^- respectively and grew for 6 d. For (**a**-**d**, **f**), Data are means ± SEM (*n* = 9). *t*-test was at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ns, not significant
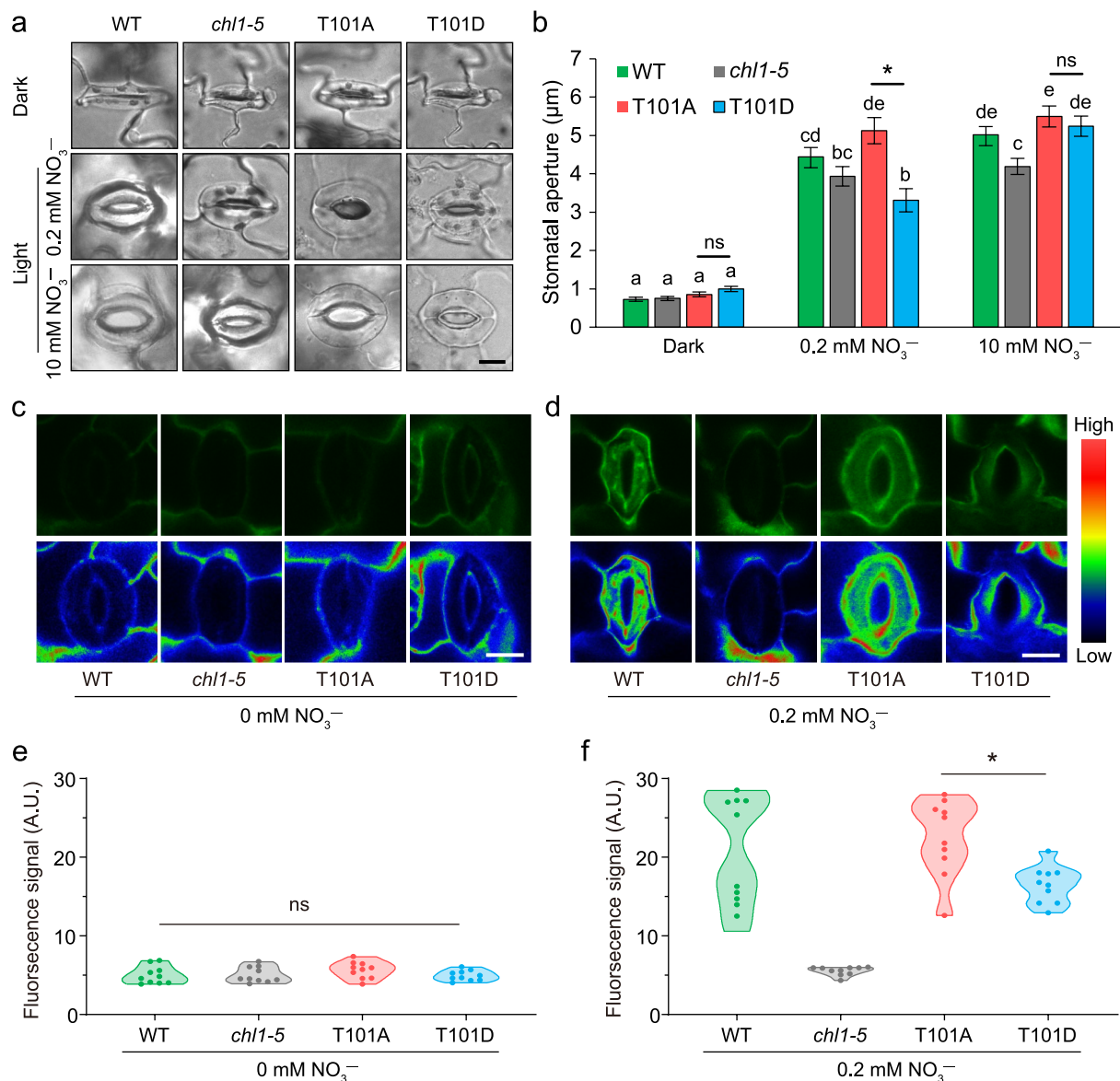
Fig. 3 The opening of stomata aperture and depolarization of membrane potential in T101D mutants were reduced compared with T101A under LN condition. (**a**, **b**) The representative stomatal morphology (**a**) and stomatal apertures (**b**) of WT, *chl1-5*, T101A and T101D plants under dark or treated with LN and HN. Bar = 10 μm. Data are means ± SEM (n = 48–73). (**c**, **d**) B-438 fluorescence from the representative guard cell of WT, *chl1-5*, T101A and T101D seedlings treated without nitrate (**c**) and with LN (**d**). Bar = 10 μm. The upper panels represent the original fluorescent signal, and the bottom panels represent the pseudocolor images. (**e**, **f**) Quantification of the B-438 fluorescence signal from the guard cells of WT, *chl1-5*, T101A and T101D seedlings treated without nitrate (**e**) and with LN (**f**). Data are means ± SEM (n = 10). For (**a-f**), 7-day-old seedlings grown on 1/2 MS medium were placed in the darkness overnight, and then exposed to 0 mM, 0.2 mM (LN) and 10 mM (HN) NO_3^- with light. For (**b**, **e** and **f**), values followed by different letters indicate significant differences (one-way ANOVA with Duncan multiple comparisons test, $P < 0.05$), t-test was at *$P < 0.05$ and ns, not significant

Therefore, we further examined the K^+ flux in WT, *chl1-5*, T101A, and T101D guard cells after exposure to 0.2 mM NO_3^-. Except for *chl1-5* mutants, WT, T101A, and T101D seedlings showed considerable K^+ influx into the guard cell (Fig. 4e, f). Notably, the mean

K^+ influx in T101A guard cells was significantly higher than that in WT and T101D respectively (Fig. 4f). These data suggest that the phosphorylation of NRT1.1 influences the K^{++} influx of guard cell in response to LN treatment.

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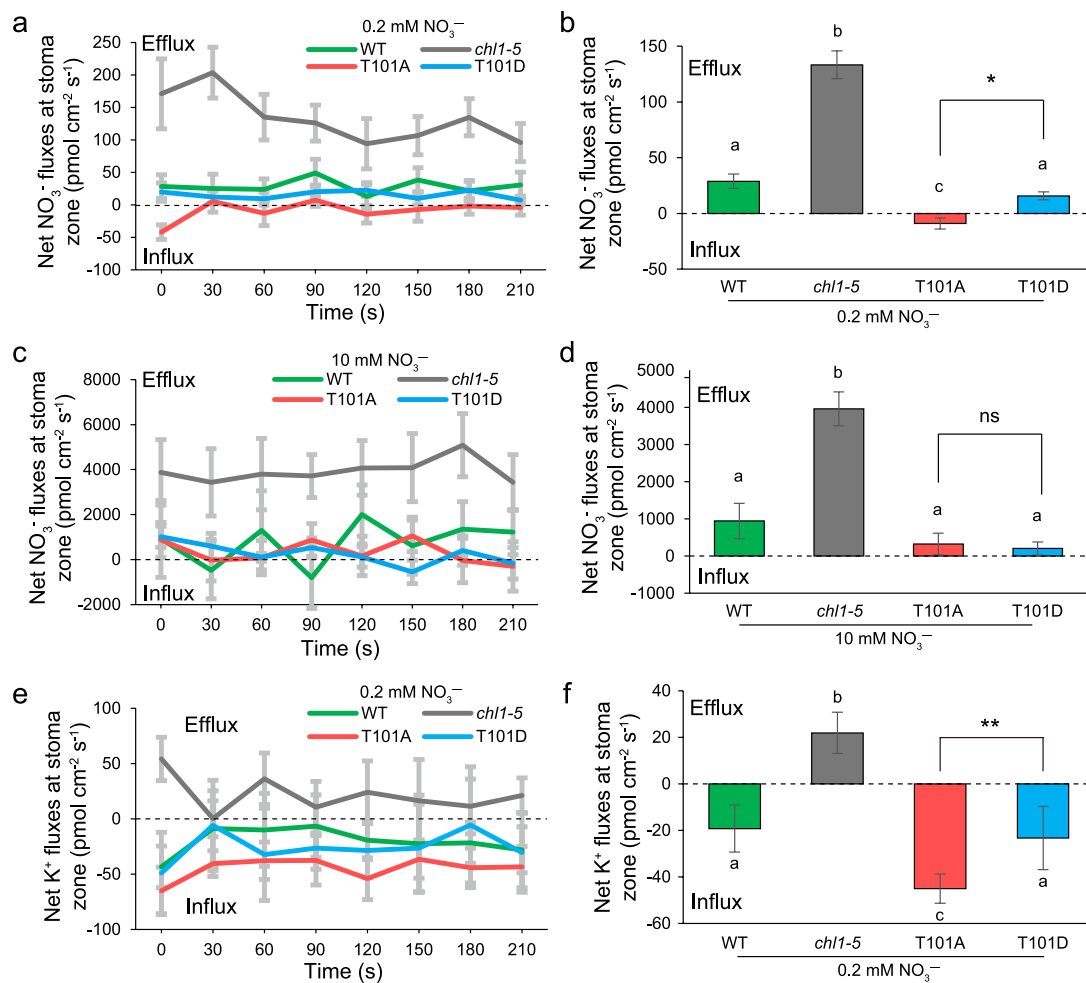
Fig. 4 T101D mutants showed significantly less NO_3^- and K^+ influx in the guard cells than T101A under LN condition. (**a**, **c**) Net nitrate flux profile of WT, *chl1-5*, T101A and T101D seedlings grown under LN (**a**) and HN (**c**) conditions. Data were recorded every 5 s. For better visualization, six time points (5 s) were aggregated into one for plotting (30 s). Data are means ± SEM (*n* = 6). (**b**, **d**) The average net nitrate flux of WT, *chl1-5*, T101A and T101D seedlings under LN (**b**) and HN (**d**) conditions. Data are means ± SEM (*n* = 48). (**e**) Net potassium flux profile of WT, *chl1-5*, T101A and T101D seedlings grown under LN condition. Data were recorded every 5 s. For better visualization, six time points (5 s) were aggregated into one for plotting (30 s). Data are means ± SEM (*n* = 6).(**f**) The average net potassium flux of WT, *chl1-5*, T101A and T101D seedlings under LN condition. Data are means ± SEM (*n* = 48). For (**a**-**f**), 4-week-old seedlings were placed in the darkness overnight. Detached leaves were exposed to LN or HN with light for 2 h before NMT analysis. For (**b**, **d** and **f**), values followed by different letters indicate significant differences (one-way ANOVA with Duncan multiple comparisons test, *P* < 0.05), *t*-test was at *******P* < 0.01, *P < 0.05 and ns, not significant
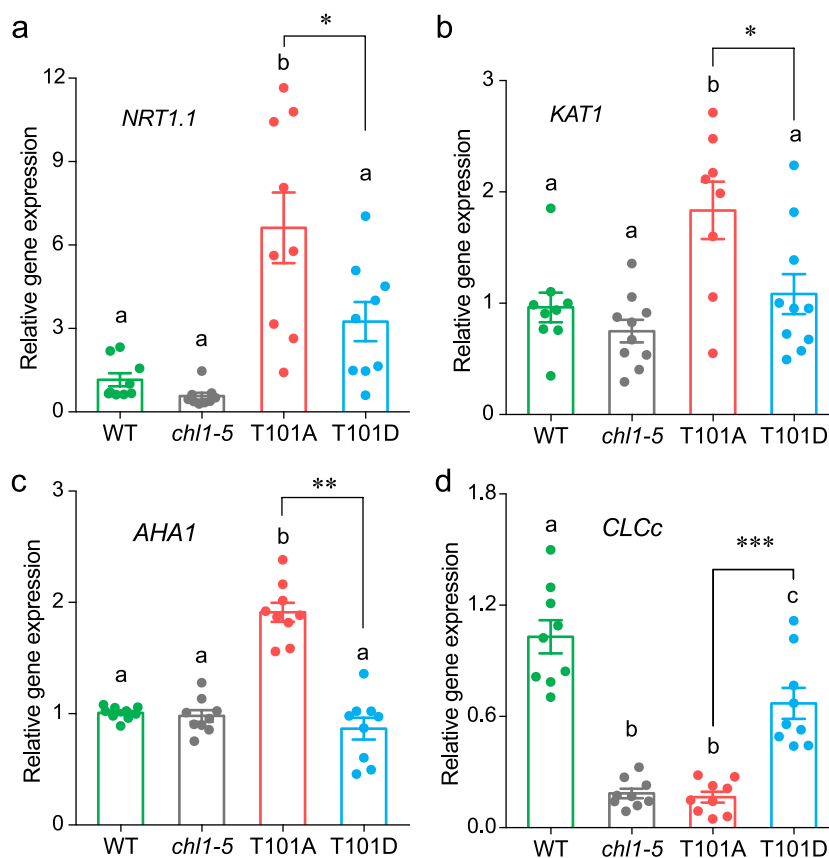
T101D mutants showed lesser NRT1.1, KAT1 and AHA1 expression as well as greater AtCLCc expression under LN condition

Since the lack of plasmodesmata in mature guard cells, the majority of the solute flow events should occur via the ion channels and transporters located at the PM [4]. Due to the difference of NO_3^- or K^+ flux between T101A and T101D under LN condition, we monitored the expression pattern of *NRT1.1* and *KAT1*, the main transporter for NO_3^- and inward channel for K^+ in guard cells [4, 6, 9, 19, 21]. In response to 0.2 mM NO_3^-

induction, the expression level of *NRT1.1* and *KAT1* in T101A was dramatically higher than that in WT, *chl1-5* and T101D seedlings (Fig. 5a, b), which was in line with the tendency of NO_3^- or K^+ flux (Fig. 4).

PM H$^+$-ATPase AHA1 plays a major role in the light-induced stomatal opening through the H$^+$ efflux from the cytosol [22]. As shown in Fig. 5c, similar to *NRT1.1* and *KAT1*, the transcriptional level of *AHA1* in T101A was 1.9-, 1.9-, and 2.4-fold greater than that of WT, *chl1-5*, and T101D respectively when 0.2 mM NO_3^- supplied, implying a defaced H$^+$ efflux progress affected by phosphorylated NRT1.1 under LN condition.

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Fig. 5 T101D mutants showed significantly less *NRT1.1*, *KAT1* and *AHA1* expression as well as obviously greater *AtCLCc* expression than T101A under LN condition. Expression level of *NRT1.1* (**a**), *KAT1* (**b**), *AHA1* (**c**) and *AtCLCc* (**d**) in WT, *chl1-5*, T101A and T101D under LN condition. 10-day-old seedlings grown on basic medium containing 0.2 mM NO_3^- were harvested for total RNA extraction and qRT-PCR assay. Data are means ± SEM (*n* = 9). Values followed by different letters indicate significant differences (one-way ANOVA with Duncan multiple comparisons test, *P* < 0.05), *t*-test was at ***P < 0.001, **P < 0.01, and *P < 0.05

At CLCc, which primarily functions in the transport of Cl^- into the vacuole, is involved in the abscisic acid (ABA)-regulated stomatal movement [23]. Interestingly, we found an opposite expression pattern of *AtCLCc* in comparison to that of *NRT1.1*, *KAT1* and *AHA1*. The T101A seedlings exhibited severe compromised *AtCLCc* expression compared with T101D and WT plants (Fig. 5d), which might reduce the sensitivity to ABA-induced stomatal closure and then lead to the increased stomatal aperture.

Discussion

Increasing evidence has illustrated that the phosphorylation state of NRT1.1^{T101} affects nitrate transport and signaling as well as auxin influx in the root system. Considering that nitrate can induce stomata opening in a NRT1.1-dependent manner [6], we here focused on the role of NRT1.1 phosphorylation in stomatal movement and drought tolerance. Our results reveal that, under LN condition, phosphorylated NRT1.1 decelerate the

expression of *NRT1.1*, *KAT1* and *AHA1*, thus reducing the ion influx and membrane depolarization, which further contributes to stomata closure and drought resistance (Fig. 6).

Stomatal movement, which is critical for water loss and drought tolerance, has tight relevance with flux of various ions (such as H^+, K^+, Cl^- and NO_3^-) mediated by corresponding channels and transporters. In this study, we found that the expression of *NRT1.1*, *KAT1* and *AHA1* in T101D mutants was significantly lower than that in T101A whereas *AtCLCc* expression was obviously higher (Fig. 5), possibly indicating the inactivation of H^+ pumping out, K^+ and NO_3^- uptake as well as activation of Cl^- translocation into vacuole. These expression patterns were consistent with and well supported the observed difference of NO_3^- and K^+ flux between T101D and T101A plants by NMT analysis. It would make sense that anion channels mediating stomatal closure might have a preference for nitrate under LN condition. Interestingly, we showed here

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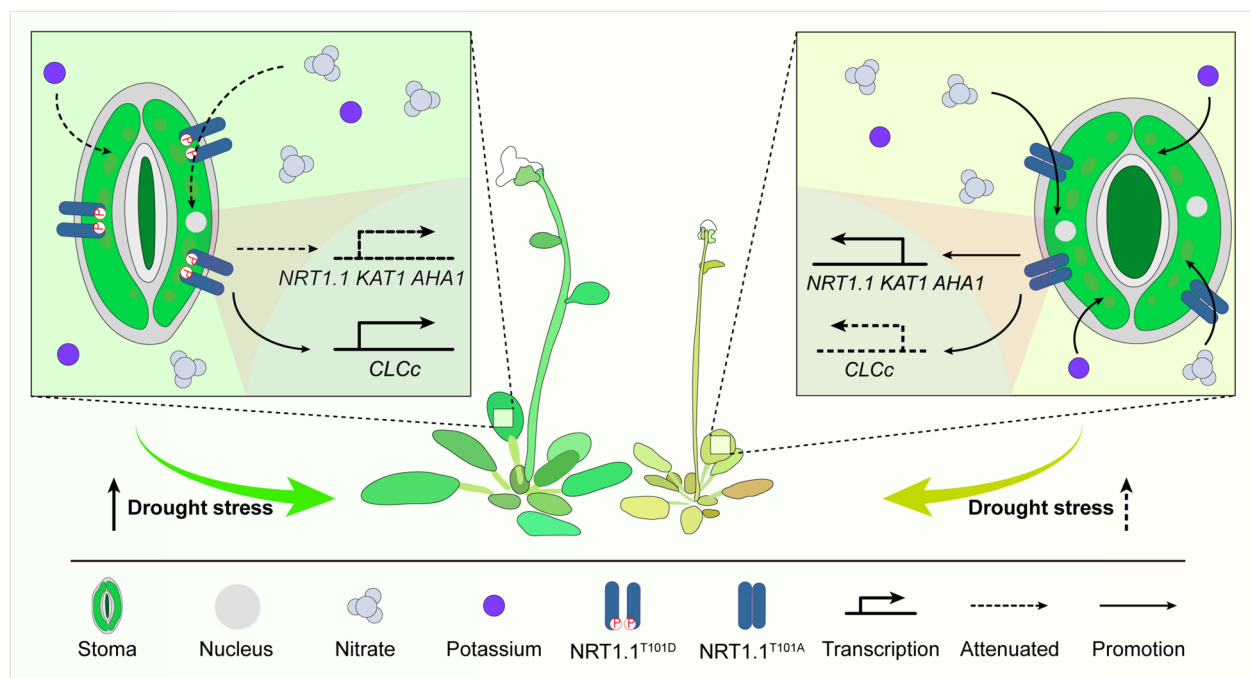
Fig. 6 Schematic diagram of NRT1.1-phosphorylation-regulated stomatal opening and drought resistance under LN condition in plants. The phosphorylation of NRT1.1 at the T101 is vital for regulating stomatal movement and plant drought tolerance. It influences the expression of ion transporters as well as the corresponding ion influx across the PM, thereby modulating membrane depolarization of guard cells and stomatal aperture. The stomatal behavior eventually contributes to the drought tolerance of plants under LN condition

that HN induced both T101D and T101A mutants to a large stomatal aperture (Fig. 3a, b), which might not be due to the increased nitrate uptake because T101D showed low nitrate transport ability both under LN and HN conditions (Fig. 4) [24]. *AHA1* expression of T101D mutants was found as a similar level as T101A mutants under HN (Fig. S5), indicating that the large stomatal apertures were probably caused by activated H^+-ATPase instead of nitrate influx.

In guard cells, NRT1.1 functions as an electrogenic nitrate transporter that depolarizes the PM during nitrate uptake [6, 18, 25]. Thus, changes in membrane potential are indicative of nitrate uptake into the cell. Further confirmation that phosphorylated NRT1.1 defects in stomata opening was obtained by examining changes in guard cells membrane potential. Under LN, the average depolarization events of T101D are significantly lower than T101A, and even visible lower than WT (Fig. 3d, f). Net nitrate influx measurement by NMT technique also proved that T101A possesses the higher ability of nitrate transport than that of WT and T101D in response to LN treatment (Fig. 4a, b). The results of K^+ uptake detection were analogous to nitrate influx assay that the reduced K^+ uptake was found in T101D mutants compared with T101A (Fig. 4e, f). Together, our data reveal a crucial role of phosphorylated NRT1.1 in

nitrate and K^+ influx which could function in reduced stomatal aperture contributing to water retention and drought resistance.

In this study, we found that both detached leaves and intact plants of T101D mutants present a remarkably decreased water loss rate (Fig. 2), which enabled the T101D mutant with high SR compared with T101A and WT plants during desiccation under LN (Fig. 1). The slower water loss and higher SR of T101D mutants are mainly attributed to their smaller stomatal apertures in comparison to T101A and WT under LN (Fig. 3a, b). Collectively, our data support a role for phosphorylation forms of NRT1.1 in stomata movement and drought tolerance.

Conclusions
Based on our findings, the phosphorylation of NRT1.1 at the T101 plays a crucial role in regulating stomatal opening and plant drought tolerance under LN condition. The differential expression patterns of *NRT1.1*, *KAT1*, *AHA1* and *AtCLCc* between T101A and T101D mutants affected transmembrane ion transport in guard cells. Mutants with phosphomimetic modifications at T101 exhibited decreased nitrate and potassium influx as well as reduced membrane depolarization, ultimately inducing stomatal closure to reduce water loss and

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enhance drought resistance. These findings underscore the significance of phosphorylated NRT1.1 in regulating stomatal movement and propose a potential strategy for improving the adaptability of plants under low nutrient and drought conditions.

Abbreviations

AHA1	Arabidopsis H^+ ATPase 1
AtCLCc	Arabidopsis thaliana tonoplast-located CHLORIDE CHANNEL C
Cl^-	Chloride
HN	High nitrate
K^+	Potassium
KAT1	Hyperpolarization-gated K^+ channel 3-KETO-ACYL-COA THIOLASE 1
LN	Low nitrate
LR	Lateral root
NMT	Non-invasive micro-test technology
NO_3^-	Nitrate
NRT1.1	NITRATE TRANSPORTER 1.1
PM	Plasma membrane
PNR	Primary nitrate response
qRT-PCR	Quantitative real-time PCR
SR	Survival rate
T101	Threonine residue 101
T101A	Threonine residue 101 of NRT1.1 replaced with alanine (Ala, A)
T101D	Threonine residue 101 of NRT1.1 replaced with aspartic acid (Asp, D)

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-06008-1.

Supplementary Material 1: Figure S1. Schematic diagram of *NRT1.1* gene in WT, T101A, T101D, and *chl-5* used for this study. Figure S2. T101D mutants are more drought-tolerant than T101A mutants.Figure S3. *chl1-5* mutants are less drought-tolerant than WT plants after drought stress.Figure S4. The B-438 dye fluorescence pattern of WT guard cells without nitrate or with LN treatment. Figure S5. T101D and T101A mutants showed similar *AHA1* expression levels under HN condition. Supplementary Table 1. Primers used in this study.

Authors' contribution

X.Y.S. and X.Z. designed the research. X.Z., Y.C.K., and B.D.S. performed most of the experiments; S.Y.Y. provided assistance in the phenotypic testing of plant drought resistance. W. G. assisted in capturing some confocal microscope images; Y.C.K. and B.D.S. analyzed the data and wrote the manuscript; X.Y.S and X.Z. revised the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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