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To cite this version:

Elsa Demes, Laetitia Besse, Paloma Cubero-Font, Béatrice Satiat-Jeunemaître, Sébastien Thomine, et al.. Dynamic measurement of cytosolic pH and [NO₃⁻] uncovers the role of the vacuolar transporter AtCLCa in cytosolic pH homeostasis. Proceedings of the National Academy of Sciences of the United States of America, National Academy of Sciences, 2020, 117 (26), pp.15343-15353. 10.1073/pnas.2007580117. hal-02873658

HAL Id: hal-02873658

https://hal.inrae.fr/hal-02873658

Submitted on 27 Nov 2020

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Dynamic measurement of cytosolic pH and [NO$_3$\(^{-}\)] uncovers the role of the vacuolar transporter AtCLCa in cytosolic pH homeostasis

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Edited by Julian I. Schroeder, Cell and Developmental Biology Section, Division of Biological Sciences, University of California San Diego, La Jolla, CA, and approved May 19, 2020 (received for review April 24, 2020)

Ion transporters are key players of cellular processes. The mechanistic properties of ion transporters have been well elucidated by biophysical methods. Meanwhile, the understanding of their exact functions in cellular homeostasis is limited by the difficulty of monitoring their activity in vivo. The development of biosensors to track subtle changes in intracellular parameters provides invaluable tools to tackle this challenging issue. AtCLCa (Arabidopsis thaliana Chloride Channel a) is a vacuolar NO$_3$\(^{-}/\)H$^+$ exchanger regulating stomata aperture in A. thaliana. Here, we used a genetically encoded biosensor, ClopHensor, reporting the dynamics of cytosolic anion concentration and pH to monitor the activity of AtCLCa in vivo in Arabidopsis guard cells. We first found that ClopHensor is not only a Cl$^-$ but also an NO$_3$\(^{-}\) sensor. We were then able to quantify the variations of NO$_3$\(^{-}\) and pH in the cytosol. Our data showed that AtCLCa activity modifies cytosolic pH and NO$_3$\(^{-}\). In an AtCLCa loss of function mutant, the cytosolic acidification triggered by extracellular NO$_3$\(^{-}\) and the recovery of pH upon treatment with fusicoccin (a fungal toxin that activates the plasma membrane proton pump) are impaired, demonstrating that the transport activity of this vacuolar exchanger has a profound impact on cytosolic homeostasis. This opens a perspective on the function of intracellular transporters of the Chloride Channel (CLC) family in eukaryotes: not only controlling the intraorganlumen but also, actively modifying cytosolic conditions.

The fluxes of ions between cell compartments are driven by membrane proteins forming ion channels, exchangers, transporters, and pumps. Defects in the transport systems residing in intracellular membranes result in major physiological failures at the cellular and the whole-organism levels (1). The localization of transport systems in intracellular membranes prevents the use of in vivo electrophysiological approaches, considerably limiting our understanding of their cellular functions. Among the different families of ion transporters identified, the CLC (Chloride Channel) family, which has been widely investigated in the last decades, constitutes a group of membrane proteins present in all organisms (2). The members of the CLC family function as anion channels or anion/H$^+$ exchangers sharing a similar structural fold (3, 4). In eukaryotes, all of the CLCs localized in intracellular membranes behave as anion/H$^+$ exchangers in mammals, mutations in intracellular CLCs lead to severe genetic diseases affecting bones, kidneys, and the brain (2). In plants, CLCs regulate nutrient storage and photosynthesis and participate in drought and salt stress tolerance (5–11). In the last few decades, many studies addressed the biophysical properties of intracellular CLCs and provided a solid ground to understand the transport mechanisms of these exchangers (12–16). However, we still lack a molecular interpretation of the role of the CLC exchangers within cells, preventing a full understanding of the defects observed in organisms carrying mutations in CLC genes (2).

Plant guard cells (GCs) constitute an appropriate experimental model to unravel CLC functions at the subcellular level. In plants, GCs are specialized cells gating the stomata pores at the leaf surface. Their biological function relies on the regulation of ion transport systems residing in the plasma membrane (PM) and vacuolar membrane (VM) (17–19). The VM delimits the largest intracellular compartment of GCs, the vacuole (17, 20). Stomata control gas exchanges between the photosynthetic tissues and the atmosphere, including water loss by transpiration. Two GCs delimit the stomata pore and regulate its aperture according to environmental conditions. The regulation of the stomata pore aperture is based on the capacity of GCs to change their turgor pressure and consequently, their shape. Increase and decrease of the turgor pressure in GCs open and close the stomata, respectively. Turgor changes in GCs depend on the accumulation/release of ions into/from the vacuole. Therefore, vacuolar ion transporters are key actors of stomata responses. The identification of a growing number of ion transporters and channels that function in the VM of GCs highlighted the importance of intracellular transport systems selective for anions, such as NO$_3$\(^{-}\), Cl$^-$, and malate$^{-}$, and for cations, such as potassium (7, 8, 21–25).

Intracellular transporters are key actors in cell biological processes. Their disruption causes major physiological defects. Intracellular ion transporters are usually thought to control luminal conditions in organelles; meanwhile, their potential action on cytosolic ion homeostasis is still a black box. The case of a plant Chloride Channel (CLC) is used as a model to uncover the missing link between the regulation of conditions inside the vacuole and inside the cytosol. The development of an original live imaging workflow to simultaneously measure pH and anion dynamics in the cytosol reveals the importance of an Arabidopsis thaliana CLC, AtCLCa, in cytosolic pH homeostasis. Our data highlight an unsuspected function of endomembrane transporters in the regulation of cytosolic pH.

Author contributions: S.T. and A.D.A. designed research; E.D., L.B., P.C.-F., and A.D.A. performed research; E.D., L.B., P.C.-F., and A.D.A. analyzed data; and B.S.-J., S.T., and A.D.A. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2007580117/-/DCSupplemental.

First published June 16, 2020.
Anion channel and transporter families such as Slow Activating Anion Channels (SLAC/SLAH), Aluminum Activated Malate Transporter (ALMT), and CLC strongly influence GC function and stomata responses to environmental changes (7, 21–23, 26–29). However, the observed GC phenotypes and the biochemical characteristics of these ion transport systems can be somewhat difficult to reconcile (7, 9, 20, 21, 27, 30). The vacuolar CLC AtCLCa (Arabidopsis thaliana Chloride Channel a) is illustrative of this difficulty. AtCLCa is known to act as a 2NO₃⁻/H⁺ exchanger driving the accumulation of NO₃⁻ into the vacuole (6, 31), suggesting a role in stomata opening. However, analysis of GC responses from AtCLCa knockout plants revealed that AtCLCa is not only involved in light-induced stomata opening but also, in abscisic acid (ABA)-induced stomata closure (7). This intriguing dual role questions the molecular interpretation of the subcellular role of AtCLCa. Being anion/H⁺ exchangers, intracellular CLCs are expected to induce simultaneous modifications of [NO₃⁻], [Cl⁻], and pH in both the lumen of intracellular compartments and the cytosol. However, so far, only their role in regulating luminal-side conditions has been investigated in plants using isolated vacuoles (32) and in mammals in lysosomes and endosomes (14, 16, 33). In mammals, CLC-5 was shown to contribute to the acidification of endosomes (33), while CLC-7 activity was associated only with modest changes in lysosomal pH that could not be detected in all studies (16, 33). In both cases, the link between luminal acidification and the severe phenotypes observed in the corresponding knockout mice was not established (16, 33). In plants, no role of a CLC transporter in vacuolar pH regulation was so far demonstrated in vivo. Here, we hypothesized that AtCLCa activity affects cytosolic parameters in addition to its well-documented role in anion accumulation inside vacuoles. We therefore aimed to visualize whether the activity of an intracellular CLC like AtCLCa induces changes in the cytosolic pH and [NO₃⁻, Cl⁻] dynamics in living GCs.

In order to be able to detect simultaneously the subtle changes in cytosolic pH and anion concentration induced by the activity of an intracellular transporter, we introduced the genetically encoded biosensor ClopHensor into GCs as an experimental model. ClopHensor is a ratiometric biosensor originally developed in mammalian cells with spectroscopic properties allowing us to measure [Cl⁻] and pH in parallel (34). Our results demonstrated that ClopHensor allows simultaneous measurements of the cytosolic pH, [Cl⁻] (34), and additionally, [NO₃⁻], which is an abundant anion in plant cells. We expressed ClopHensor in the cytosolic compartment (cyt) of Arabidopsis and conducted imaging experiments on GCs to visualize the subcellular effects of the activity of the NO₃⁻/H⁺ exchanger AtCLCa in vivo. We monitored by confocal laser scanning microscope (CLSM) the changes in [Cl⁻]ₜrat or [NO₃⁻]ₜrat in parallel with pHcyt. We developed a specific image analysis workflow to measure the fluorescence ratios of interest in GCs. A comparative study between GCs from wild-type and AtCLCa knockout mutant plants shows that the vacuolar exchanger AtCLCa not only controls the kinetics of [NO₃⁻]ₜrat but also changes, actively participates in the control of pHcyt. These results highlight an unexpected role of AtCLCa in the regulation of pHcyt. Furthermore, they open a perspective on the cellular functions of intracellular transporters in GCs that might provide an integrated framework to understand the function of intracellular CLCs in other eukaryotic cells.

**Results**

**In Vitro Assays Reveal a Strong Affinity of ClopHensor for NO₃⁻**. In contrast to mammalian cells, several anionic species are present in the millimolar range in plant cells (5, 35). Therefore, we investigated the sensitivity of ClopHensor to Cl⁻, NO₃⁻, PO₄³⁻, malate²⁻, and citrate³⁻, the main anions present in the model plant Arabidopsis (5). ClopHensor was previously shown to be insensitive to SO₄²⁻, which also accumulates to millimolar levels in plant cells (34, 36). We used recombinant ClopHensor proteins bound to Sepharose beads and recorded the fluorescence upon exposure to a range of anions by CLSM after excitation at 458 nm (emission 500 to 550 nm), 488 nm (emission 500 to 550 nm), and 561 nm (emission 625 nm) (Fig. 1A). The ratio Rₐnion (F₄₈₈/F₅₆₁) was calculated from the ratio of the fluorescence intensity images after excitation at 458 nm (F₄₅₈) and 561 nm (F₅₆₁) to estimate the effect of anions on ClopHensor. (SI Appendix) has an Rₐnion calculation. No significant difference in Rₐnion was observed between the control (Rₐnion,control = 1.14 ± 0.11) and 30 mM PO₄³⁻ (Rₐnion,PO₄ = 0.88 ± 0.05), malate²⁻ (Rₐnion,malate = 0.91 ± 0.02), and citrate³⁻ (Rₐnion,citrate = 0.92 ± 0.05) (Fig. 1A). Meanwhile, we found that ClopHensor was sensitive to Cl⁻ (Rₐnion,Cl⁻ = 0.42 ± 0.03) as previously reported (34) and remarkably, also to NO₃⁻ (Rₐnion,NO₃⁻ = 0.21 ± 0.03) (Fig. 1A). ClopHensor displayed a higher affinity to NO₃⁻ (Kᵣ₊₉ₒ₉ = 5.3 ± 0.8 mM at pH 7) than to Cl⁻ (Kᵣ₊₉ₒ₉ = 17.5 ± 0.5 mM at pH 6.8) (Fig. 1B). The sensitivity range of ClopHensor was between 2 and 162 mM for Cl⁻ (at pH 6.8) and between 0.6 and 48 mM for NO₃⁻ (at pH 7) (Fig. 1B).

Notably, in the physiological range of cytosolic pH (i.e., 6.8 to 8), the Kᵣᵦₒ₉ of ClopHensor was between 5 and 25 mM (SI Appendix, Fig. S1), which is in the range of the previously reported [NO₃⁻]ₜrat values of about 5 mM (35), therefore making it suitable to monitor the dynamics of this anion. Concerning chlorides, Kᵣᵦₒ₉ of ClopHensor was between 17.5 and 163 mM (SI Appendix, Fig. S1), values that are above the reported basal [Cl⁻]ₜrat in plant cells of about 10 mM (37). To test the pH sensitivity of ClopHensor in our in vitro assays, we calculated the ratio Rₕᵦᵦ (F₄₈₈/F₅₆₁) (SI Appendix has an Rₕᵦᵦ calculation). In agreement with a previous report (34), we found a strong response of Rₕᵦᵦ to pH variations with a steep dynamic range of ninefold change between pH 6.1 and pH 7.9 and a pKᵦₕᵦ = 6.98 ± 0.09 (Fig. 1D). Neither the binding of NO₃⁻ nor that of Cl⁻ modified significantly the pH sensitivity of ClopHensor (SI Appendix, Fig. S1), confirming its robustness as a dual anion and pH biosensor.

ClopHensor Is a Robust and Sensitive Sensor of Cytosolic pH in A. thaliana GCs. We generated transgenic Arabidopsis plants (ecotype Columbia 0 [Col-0]) expressing ClopHensor in the cytosol and nucleoplasmic compartments under the control of the Ubiquitin10 promoter (pUB10:ClopHensor). The expression of ClopHensor did not affect the development of the plants, indicating that its expression did not significantly interfere with the amount of anions available in the cytosol for cellular metabolism (SI Appendix, Fig. S2). To measure the pH sensitivity of ClopHensor in living GCs, stomata from pUB10:ClopHensor were sequentially exposed to NH₄-acetate–based buffers to clamp the pHcyt at defined values between 5 and 9 (Fig. 1C and D). We found that ClopHensor sensitivities to pH in vivo and in vitro were very similar. The mean <pHcyt> calculated from each pixel in the stomata, showed that the pH titration curve of ClopHensor in GCs mirrored the in vitro assay (Fig. 1D). The pKᵦₕᵦ = 6.98 ± 0.11 and the sensitivity range of ClopHensor (between pH 6.1 and 7.9) measured in vivo matched the values measured in vitro (Fig. 1D). These findings demonstrate that 1) ClopHensor is a reliable reporter for intracellular pH changes in GCs, 2) the cytosolic environment does not affect ClopHensor properties with respect to pH, and 3) the ClopHensor sensitivity range is appropriate for measuring pHcyt in GCs.

**Settings and Design of the Experimental Workflow in GCs**. The data we obtained open the possibility of measuring the variations of [NO₃⁻]ₜrat, [Cl⁻]ₜrat, and pHcyt in vivo. This provides a unique opportunity to disclose in living cells how ion fluxes across the PM and the VM of GCs affect cytotoxic conditions. In order to
Quantify \([\text{NO}_3^-]_{\text{cyt}}, [\text{Cl}^-]_{\text{cyt}}, \text{and pH}_{\text{cyt}}\) in GCs, we optimized the fluorescence acquisition protocol in GCs expressing ClopHensor (SI Appendix, Figs. S3 and S4) and determined the temporal window to set up our experiments. First, to maximize the collected fluorescence and minimize photodamage by the laser, we selected stable transgenic lines expressing pUBI10:ClopHensor with high fluorescence in GCs after excitation at 488 nm (emission 500 to 550 nm), 488 nm (emission 500 to 550 nm), and 561 nm (emission 605 to 625 nm). Second, to quantify \([\text{NO}_3^-]_{\text{cyt}}, [\text{Cl}^-]_{\text{cyt}}, \text{and pH}_{\text{cyt}}\), we excluded the fluorescent signals emitted by chloroplasts (excitation 488 nm, emission 650 to 675 nm). Therefore, we developed an image processing workflow to accurately measure ClopHensor fluorescence in the cytosol of plant cells (SI Appendix, Fig. S4).

To derive the \([\text{NO}_3^-]_{\text{cyt}}, [\text{Cl}^-]_{\text{cyt}}, \text{and pH}_{\text{cyt}}\) in GCs, we used the calculation procedure described in Arosio et al. (34) (SI Appendix). To obtain a quantitative estimation of the changes in \([\text{NO}_3^-]_{\text{cyt}}\) and \([\text{Cl}^-]_{\text{cyt}}\) induced by the applied treatments, we determined in vivo the ratio \(R_{\text{ratio}} = F_{448}/F_{498}\). The ratio \(R_{\text{ratio}}\) is required to calculate the actual concentration of \(\text{Cl}^-\) and \(\text{NO}_3^-\) in the cytosol (SI Appendix). To this aim, we set up experimental conditions where the initial endogenous \([\text{NO}_3^-]_{\text{cyt}}\) and \([\text{Cl}^-]_{\text{cyt}}\) should be below the sensitivity threshold of ClopHensor. Selective microelectrode measurements have shown that, when plants are grown with less than 0.01 mM \(\text{NO}_3^-\) supply, the cytosolic levels are below 0.5 mM (38). Therefore, we grew pUBI10::ClopHensor plants in vitro in an \(\text{NO}_3^-\)-free medium (0 mM \(\text{NO}_3^-\) medium) and determined the whole-plant \([\text{NO}_3^-]\) and \([\text{Cl}^-]\) at different days after germination (DAG) (SI Appendix, Table S1). We found that, in these conditions, the whole-seedling endogenous content of \(\text{NO}_3^-\) and \(\text{Cl}^-\) was decreasing after germination. At DAG 14, \(\text{Cl}^-\) was no longer detectable; meanwhile, \([\text{NO}_3^-]_{\text{cyt}}\) was below the sensitivity threshold of ClopHensor (i.e., 0.6 mM at pH 7). Subsequently, based on these data, we imaged the fluorescence in stomata from pUBI10::ClopHensor plants grown in vitro for 14 d on an \(\text{NO}_3^-\)-free medium and measured a mean ratio \(R_{\text{ratio}}\) of 0.56 ± 0.07 (n = 29 stomata) (SI Appendix, Fig. S3E).

Dynamic Measurements of Cytosolic \(\text{NO}_3^-\), \(\text{Cl}^-\), and \(\text{pH}\) in Arabidopsis GCs. We challenged 14-d-old \(\text{NO}_3^-\)-starved Arabidopsis seedlings expressing ClopHensor for the simultaneous detection in GCs of \([\text{NO}_3^-]_{\text{cyt}}, [\text{Cl}^-]_{\text{cyt}}, \text{and pH}_{\text{cyt}}\) changes upon extracellular \(\text{NO}_3^-\) or \(\text{Cl}^-\) supply/removal (Fig. 2). The experimental design was based on the application of different extracellular conditions in a sequence of five steps (Fig. 2). GCs were 1) perfused with \(\text{NO}_3^-\)-free medium to determine the ratio \(R_{\text{di}}\) for each stomata; 2) exposed to 30 mM KNO3 to observe [\(\text{NO}_3^-\)] \(\text{cyt}\) changes; 3) washed out with \(\text{NO}_3^-\)-free medium; 4) exposed to 30 mM KCl to observe [\(\text{Cl}^-\)] \(\text{cyt}\) changes; and 5) washed out again with \(\text{NO}_3^-\)-free medium. We applied 30 mM KNO3 or KCl as these concentrations are commonly used in stomata aperture assays (8, 39). To perform a full experiment, we imaged GCs for 190 min, and each stomata was imaged every 4 min with sequential excitation at 561, 488, and 458 nm. Fluorescence intensity recorded in \(\text{NO}_3^-\)-free medium was not altered after 190 min of illumination, indicating that ClopHensor was not significantly affected by photobleaching over the whole duration of the experiment (SI Appendix, Figs. S5 and S6). Raw data suggested striking variations of the mean fluorescence intensity recorded after excitation.
Similar results were obtained when stomata were exposed to KCl [NO3]-free medium (SI Appendix, Fig. S5). Ratiometric images for RpH and Rclca were established from the fluorescence intensity images (Fig. 2A and C). The ratiometric maps for RpH and Rclca were then used to compute the mean pHcyt (Fig. 2B) and the mean Rclca in the presence of extracellular NO3- and Cl- for each cell (Fig. 2D). The results show that, differently from our observations with NO3-, Rclca does not change significantly upon addition of Cl-, suggesting that [Cl]-cyt was below the range of sensitivity of ClopHensor. In addition, the comparison of pHcyt and Rclca changes in the presence of extracellular NO3- during the experiment suggests a link between NO3- transport and pH modification (Fig. 2B and D). Initially (step 1), in the NO3- free medium, the pHcyt was 7.01 ± 0.19. Within 35 min, it increased and stabilized to 7.17 ± 0.18, while Rclca was constant (Fig. 2D). Upon addition of 30 mM extracellular KNO3 (step 2), the Rclca decreased from a mean value in 0 mM NO3- of 0.49 ± 0.08 to a value of 0.25 ± 0.03. The calculation of the [NO3]-cyt shows that it increased from an initial value of 0.74 ± 0.25 to 4.91 ± 0.40 mM. In parallel, the pHcyt decreased to 6.78 ± 0.04. Both pHcyt and Rclca reached a plateau within 20 to 30 min, suggesting a coordination between the two parameters. At step 3, unexpectedly both pHcyt and Rclca dropped back to their initial values in less than 4 min after removal of KNO3. Finally (step 4), when the stomata were exposed to 30 mM KCl, a modest and not significant (P = 0.17, n = 6) decrease from pHcyt = 7.17 ± 0.20 to pHcyt = 7.05 ± 0.40 was observed, with a rate of pH decrease lower than with 30 mM KNO3 (Fig. 2B). Similar results were obtained when stomata were exposed to KCl only (SI Appendix, Fig. S7).

As a whole, these data demonstrate that ClopHensor enables us to simultaneously monitor in vivo the variations in [Cl]-cyt or [NO3]-cyt and pHcyt at a cellular resolution. In the conditions tested, [Cl]-cyt was below the limit of detection of ClopHensor for Cl- (i.e., 2 mM) (Fig. 2D). This suggests that in our experimental setting, ClopHensor was measuring essentially cytosolic NO3- variations. Notably, cytosolic NO3- and pH changes appear to be concerted, suggesting that they are governed by a common mechanism.

**AtCLCa Accounts for Cytosolic Acidification in Response to NO3-.** The finding that ClopHensor can measure the dynamic changes of [NO3]-cyt and pHcyt in GCs opens the possibility to visualize the activity of intracellular ion transport systems in living cells. We therefore used this sensor to address the role of the vacuolar 2NO3-/H+ exchanger AtCLCa in cytosolic NO3- and pH homeostasis. AtCLCa is known to mediate the uptake of NO3- into the vacuole driven by H+ extrusion into the cytosol (6, 40). Therefore, based on its biophysical properties, AtCLCa may be involved in the [NO3]-cyt and pHcyt responses measured in Fig. 2. To assess this possibility, we generated clca-3 knockout mutant plants expressing ClopHensor by crossing clca-3 with a wild-type pUB110:ClopHensor line. Patch-clamp experiments performed on vacuoles isolated from the wild type and clca-3 pUB110:ClopHensor confirmed that clca-3 plants expressing pUB110:ClopHensor were defective in vacuolar NO3- transport activity (SI Appendix, Fig. S8). We then compared the dynamic changes of [NO3]-cyt and pHcyt in stomata of 14-d-old nitrate-starved seedlings from wild-type and clca-3 pUB110:ClopHensor plants (Figs. 3 and 4). Since AtCLCa is highly selective for NO3- over Cl-, we performed experiments applying extracellular KNO3 only.

**Fig. 2.** ClopHensor reveals the dynamics of cytosolic pH, NO3-, and Cl- in Arabidopsis stomata. Epidermal peels from plants grown in vitro for 14 d in NO3- free media were imaged (SI Appendix). (A and C) Representative false color ratio images of RpH (A) and Rclca (C) at different time points of a stomata sequentially exposed to NO3- free medium (0 mM NO3-), 30 mM KNO3, and 30 mM KCl. Gray areas, localization of chloroplasts subtracted during the analysis. (Scale bars: 5 μm.) (B) pHcyt was quantified at each time point from the corresponding RpH images. (D) Quantification of the Rclca in the cytosol of GCs. (B and D) pHcyt (B) and Rclca (D), indicating [NO3]-cyt and Cl- variations. Horizontal error bars represent the time interval of 4 min for the sequential imaging of stomata. Data represent mean values ± SD (n = 6). SI Appendix, Fig. S4 shows the workflow for the calculation of pHcyt (B) and Rclca (D). Vertical dotted lines indicate changes of extracellular conditions. The horizontal dashed line (B) serves as a reference for pH 7.2.
Again, we designed experiments divided in five steps. GCs from the wild type and clca-3 were 1) perfused with NO$_3^-$-free medium to establish the ratio $R^0$ of each stomata; 2) perfused with 10 mM KNO$_3$; 3) washed out with NO$_3^-$-free medium; 4) perfused with 30 mM KNO$_3$; and 5) washed out with NO$_3^-$-free medium.

Application of this five-step protocol to wild-type pUBI10:ClopHensor GCs showed that [NO$_3^-$]$_{cyt}$ varies according to the applied extracellular KNO$_3$ concentration. We calculated the [NO$_3^-$]$_{cyt}$ to be 1.64 ± 0.32 and 4.74 ± 1.52 mM in 10 and 30 mM KNO$_3$, respectively ($n$ = 8) (Fig. 3 A, B, and E). In the presence of 10 mM KNO$_3$, the [NO$_3^-$]$_{cyt}$ reached a plateau in less than 4 min (Fig. 3 B). However, in the presence of 30 mM KNO$_3$ in the extracellular medium, the [NO$_3^-$]$_{cyt}$ rose progressively with a time constant of $\tau$ = 15 ± 3 min (Fig. 3 B). Interestingly, GCs maintained an [NO$_3^-$] gradient between the apoplast and the cytosol of about sixfold when either 10 or 30 mM KNO$_3$ was applied. In all cases, upon washout with NO$_3^-$-free medium, the [NO$_3^-$]$_{cyt}$ dropped back to concentrations close to the limit of detection within 4 min. In clca-3 pUBI10:ClopHensor GCs, the [NO$_3^-$]$_{cyt}$ behaved similarly to wild-type plants upon exposure to 10 mM KNO$_3$, reaching 2.24 ± 1.47 mM ($n$ = 15) (Fig. 3 C–E). However, in contrast with the wild type, [NO$_3^-$]$_{cyt}$ increased faster, reaching a plateau in less than 4 min in clca-3 ($\tau$ < 3 min) compared with about 30 min in wild-type pUBI10:ClopHensor GCs (Fig. 3 D). These data are in agreement with the involvement of the AtCLCa exchanger in buffering cytosolic NO$_3^-$.
dynamics in the wild type and clca-3 were markedly different when extracellular KNO₃ was applied (Fig. 4 A–D). In wild-type GCs, the pHₜₚt stabilized at 6.89 ± 0.05 (n = 8) at the beginning of the experiments. Then, exposure to 10 mM KNO₃ induced an initial slight pHₜₚt increase followed by a progressive and modest acidification of the cytosol. Washing out with NO₃⁻-free medium provoked a fast increase of the pHₜₚt to 7.21 ± 0.12 (n = 8). Then, upon perfusion with 30 mM KNO₃, a progressive and marked acidification to pH 6.87 ± 0.13 (n = 8) was observed within 4 min. In clca-3 pUBI10:ClopHensor GCs, a modest pHₜₚt acidification was observed upon exposure to 10 mM KNO₃, as in the wild type. However, this pHₜₚt decrease was not statistically significant in clca-3 plants (Fig. 4E). Remarkably, the perfusion of 30 mM KNO₃, which induced a marked acidification in wild-type GCs, did not induce any decrease of pHₜₚt in clca-3 GCs: pHₜₚt remained stable at pH ~ 7.3 (n = 15) (Fig. 4B and D). To exclude an effect of the sequence of KNO₃ application, we inverted step 2 and step 4 in the perfusion protocol and obtained the same results (SI Appendix, Fig. S9). These findings show that the presence of the 2NO₃⁻/1H⁺ exchanger AtCLCa in the VM is associated with the pHₜₚt modification detected in wild-type GCs upon perfusion with 30 mM KNO₃, suggesting a role of AtCLCa in the regulation of pHₜₚt. We tested whether the application of KNO₃ has an effect on stomata aperture at a whole-leaf level and performed leaf gas exchange measurements on detached leaves (SI Appendix, Fig. S10) (41, 42). In these experiments, we applied KNO₃ at the leaf petiole, and we detected an increase of stomata conductance that was similar in the wild type and clca-3 (SI Appendix, Fig. S10 and Table S4). The similar behavior of the wild type and clca-3 when

Fig. 4. The vacuolar NO₃⁻/H⁺ exchanger AtCLCa regulates pHₜₚt in Arabidopsis stomata. Epidermal peels from plants grown in vitro for 14 d in NO₃⁻-free media were imaged (SI Appendix). (A and C) Representative false color ratio images of RpH from wild-type (A) and clca-3 (C) stomata at different time points. Stomata were sequentially exposed to 0, 10, and 30 mM KNO₃ (horizontal bar in Upper). Gray areas, localization of chloroplasts subtracted during the analysis. (Scale bars: 5 µm.) (B and D) pHₜₚt (mean ± SD) at each time point in wild-type (B; n = 8) and clca-3 stomata (D; n = 15). Horizontal error bars represent the time interval of 4 min for the sequential imaging of stomata. Vertical dotted lines, changes of extracellular conditions. Horizontal dashed lines indicate pH 7.2. Black arrows, time points used for the box plot analysis in E. (E) Box plots of the pHₜₚt at different time points (black arrows in B and D). Brackets indicate statistically significant differences. Blue boxes, the wild type (n = 17); red boxes, clca-3 (n = 15). Whiskers show the 10 to 90% percentiles. Crosses indicate the means.
KNO₃ is applied converges with the finding that at a cellular level AtCLCa does not determine the steady-state [NO₃⁻][cyt] (Figs. 3 and 5). The subsequent application of 50 μM ABA on detached leaves induced a similar decrease of the stomata conductance in both the wild type and clca-3 (SI Appendix, Fig. S10 and Table S4). These results with detached leaf gas exchange measurements do not correlate with the observations made on stomata from isolated epidermis from clca knockout (7). Such discrepancy between experimental methods and conditions has been reported as well for several well-known knockout mutants involved in ABA signaling and stomata regulation such as, for example, slac1, abi1, and abi2 (41).

AtCLCa Is Involved in pH Homeostasis upon Treatment of GCs with Fusicoccin. The results obtained upon treatment with extracellular KNO₃ indicated that AtCLCa may be an important player in pHcyt homeostasis (Figs. 3 and 4). To test whether AtCLCa influences pHcyt regulation independently of the addition of its anion substrates, NO₃⁻ and Cl⁻, we investigated its role in response to the fungal toxin fusicoccin, which triggers stomata opening through a robust activation of the PM H⁺/pump (43, 44). In these experiments, we used stomata from plants grown in soil, and since we could not control the initial cellular [NO₃⁻] and [Cl⁻], we quantified the changes in pHcyt and [NO₃⁻][cyt] in GCs as ΔRpH/RpH,i and ΔRanion/Ranion,i (Fig. 5 and SI Appendix). Positive values of ΔRpH/RpH,i and ΔRanion/Ranion,i denote cytosolic alkalinization and increase in [NO₃⁻][cyt], respectively. To correlate the changes in pHcyt or [NO₃⁻][cyt] with the opening of stomata, we started the experiments with closed stomata at the end of the dark period (Fig. 5). Thus, epidermal peels from the wild type and clca-3 were prepared 1 h before the onset of light. After incubation under the microscope for 20 min in a buffer containing 10 mM KNO₃ at pH 5.7, 10 μM fusicoccin was applied for total time of 130 min. Stomata were imaged every 4 min, and we measured pore aperture, RpH,i and Ranion in each stomata (Fig. 5). Fusicoccin induced a significantly lower opening in clca-3 (1.8 μm ± 0.1 at 152 min, n = 15) compared with wild-type (2.6 μm ± 0.2 at 152 min, n = 14) stomata (Fig. 5 A and B), in agreement with previous results showing that light-induced stomata opening is reduced in clca knockout mutants (7). In wild-type stomata, fusicoccin induced a rapid increase of pHcyt, leading to a ΔRpH/RpH,i = 0.12 ± 0.02 as early as 4 min after treatment (n = 14) (Fig. 5C). Then, pHcyt slowly recovered to almost reach its initial value after 120 min (ΔRpH/RpH,i = 0.03 ± 0.02, n = 15) (Fig. 5C). Notably, wild-type stomata not treated with fusicoccin did not open and did not exhibit a significant increase of the
ΔR_{pH}/R_{pH,1} (n = 5) (Fig. 5C). In clca-3 stomata, fusicoccin induced a rapid increase of pH\textsubscript{cyt} with a ΔR_{pH}/R_{pH,1} = 0.10 ± 0.01 after 4 min (n = 14) (Fig. 5D) as in the wild type. However, in contrast with the wild type, pH\textsubscript{cyt} did not recover its initial value in clca-3 stomata, even after 120 min (ΔR_{pH}/R_{pH,1} = 0.09 ± 0.01, n = 15) (Fig. 5D). The rapid increase in pH\textsubscript{cyt} observed after fusicoccin treatment (Fig. 5C and D) is likely due to the activation of the PM H\textsuperscript{+} pumps that are extruding H\textsuperscript{+} in the apoplast (43, 44). In clca-3, the absence of an NO\textsubscript{3}\textsuperscript{-}/H\textsuperscript{+} antiporter pumping H\textsuperscript{+} from the vacuole into the cytosol accounts for the defect in pH\textsubscript{cyt} recovery after fusicoccin-induced alkalinization (6). This result shows that the transport activity of AtCLCa in the VM contributes to the recovery after the cytosolic pH increase induced by fusicoccin. Interestingly, the quantification of ΔR_{\text{ion}}/R_{\text{ion,1}} in the wild type...

![Diagram](image)

**Fig. 6.** A vacuolar exchanger modifies cytosolic homeostasis in *Arabidopsis* stomata. Illustration recapitulating the impact of the activity of AtCLCa on [NO\textsubscript{3}\textsuperscript{-}]\textsubscript{cyt} and pH\textsubscript{cyt} homeostasis in GCs. (A) In the presence of 30 mM KNO\textsubscript{3}, NO\textsubscript{3}\textsuperscript{-} enters the cell via NO\textsubscript{3}\textsuperscript{-} transporters and channels residing in the PM. In the wild type (Left), the vacuolar AtCLCa exchanger (shown in red) pumps NO\textsubscript{3}\textsuperscript{-} into the vacuole, slowing down [NO\textsubscript{3}\textsuperscript{-}]\textsubscript{cyt} increase. In the absence of AtCLCa (Right), [NO\textsubscript{3}\textsuperscript{-}]\textsubscript{cyt} stabilizes in less than 4 min. (B) In the presence of 30 mM KNO\textsubscript{3}, the transport activity of AtCLCa releases H\textsuperscript{+} in the cytosol, inducing an acidification in wild-type GCs (Left). In the absence of AtCLCa, the cytosolic acidification does not occur (Right). (C and D) Fusicoccin triggers stomata opening, activating the PM H\textsuperscript{+}-ATPase (shown in red). (C) During opening, a progressive increase of [NO\textsubscript{3}\textsuperscript{-}]\textsubscript{cyt} reaches higher levels in the wild type (Left) than in clca-3 (Right). (D) Fusicoccin induces an increase of pH\textsubscript{cyt} in both the wild type (Left) and clca-3 mutant (Right). Notably, in the wild type, within 130 min the pH\textsubscript{cyt} recovers to the initial value (Left). Differently, in clca-3, the pH\textsubscript{cyt} did not recover its initial value (Right).
showed an increase in $[\text{NO}_3^-]_{\text{cyt}}$ over the time of the experiment independently of fusicoccin application (Fig. 5E). Therefore, increased $[\text{NO}_3^-]_{\text{cyt}}$ does not seem to determine stomata opening. Intriguingly, the rate of $[\text{NO}_3^-]_{\text{cyt}}$ increase was significantly lower in clca-3 than in the wild type (Fig. 5F). This is opposite to what one would expect, as AtCLCa removes $\text{NO}_3^-$ from the cytosol to store it in the vacuole. This surprising result suggests that a more complex regulation is involved, such as a feedback of the $\text{NO}_3^-$ store in the vacuole. This result is supported by our observation that ClpHensor sensitivity to $[\text{NO}_3^-]_{\text{cyt}}$ was lower in clca-3 compared with the wild type (Fig. 2A) and that ClpHensor is sensitive not only to $\text{Cl}^-$ but also, to $\text{NO}_3^-$, while it is insensitive to $\text{PO}_4^{3-}$, malate$^{2-}$, and citrate$^{3-}$ at the tested concentrations. Furthermore, ClpHensor sensitivity to $\text{NO}_3^-$ is even higher than that to $\text{Cl}^-$ (Fig. 1). The analysis of the $[\text{NO}_3^-]_{\text{cyt}}$, $[\text{Cl}^-]_{\text{cyt}}$, and $p_{\text{Hcyt}}$ in living GCs demonstrated that ClpHensor is able to report dynamic changes of these parameters (Figs. 3–5). Interestingly, the cytosolic $[\text{NO}_3^-]_{\text{cyt}}$ we estimated is in the same range as those previously reported in other cell types with selective microelectrodes (35, 48, 49). The agreement between our data and previous reports demonstrates the robustness of ClpHensor to measure $[\text{NO}_3^-]_{\text{cyt}}$ in Arabidopsis GCs. Concerning pH, ClpHensor displays a steep dynamic range fitting cytosolic conditions (Figs. 1, 2, and 4). The steepness of the pH response is particularly valuable to resolve subtle pH changes. The properties of ClpHensor for pH measurements match those of other pHluorin-derived pH sensors used previously to measure $p_{\text{Hcyt}}$ in plant cells (50, 51). Overall, our results demonstrate that ClpHensor can be used to measure $[\text{NO}_3^-]_c$ and pH in GCs. Other $\text{NO}_3^-$ biosensors have been developed, such as NiTrak, which allows monitoring the activity of the nitrate transporter NRT1.1/NPF5.6 (52), and sNOOOpy, a nitrate/nitrite biosensor that has not been tested in plants yet (53). However, ClpHensor is the first biosensor able to report $[\text{NO}_3^-]_c$ in the cytosol of plants in parallel with pH. Given the link between anion and H$^+$ transport in plant cells, this dual capacity of ClpHensor is particularly relevant.

**A Vacuolar CLC Is Involved in Cytosolic Ion Homeostasis.** To reveal the impact of the activity of the vacuolar transporter AtCLCa, we challenged stomata of 14-d-old nitrate-starved seedlings with different extracellular media applied in a defined sequence (Figs. 2–4). Starting from an initial condition with no $\text{NO}_3^-$ or $\text{Cl}^-$ in the extracellular medium and within the GCs, we applied different KNO$_3$ and KCl-based media. In our conditions, $[\text{Cl}^-]_{\text{cyt}}$ was below the sensitivity range of ClpHensor. However, we obtained a remarkable result: $[\text{NO}_3^-]_{\text{cyt}}$ in GCs can undergo rapid variations (Figs. 2 and 3). To our knowledge, such variations of $[\text{NO}_3^-]_{\text{cyt}}$ have not been described so far. Former reports available from root epidermal cells or mesophyll protoplasts suggested that $[\text{NO}_3^-]_{\text{cyt}}$ was stable, at least in the short term (35, 49). These studies were using invasive approaches without challenging cells with modification of the extracellular ion concentrations, possibly explaining why $[\text{NO}_3^-]_{\text{cyt}}$ changes were not observed. Interestingly, our findings show that $[\text{NO}_3^-]_{\text{cyt}}$ can change rapidly, within minutes (Figs. 2 and 3). This supports the hypothesis that $[\text{NO}_3^-]_{\text{cyt}}$ variations may act as an intracellular signal. A role of $[\text{NO}_3^-]_{\text{cyt}}$ to adjust cell responses to external nitrogen supply has been previously proposed (48, 54). A second remarkable observation we made is a progressive acidification of the cytosol in parallel with the $[\text{NO}_3^-]_{\text{cyt}}$ increase. Conversely, $[\text{NO}_3^-]_{\text{cyt}}$ decrease is paralleled by a rapid $p_{\text{Hcyt}}$ increase (Figs. 2–4 and 6). These findings clearly show a link between $[\text{NO}_3^-]_{\text{cyt}}$ and $p_{\text{Hcyt}}$ changes and suggest a common molecular mechanism underlying $\text{NO}_3^-$ and pH variations.

The detected changes in $p_{\text{Hcyt}}$ and $[\text{NO}_3^-]_{\text{cyt}}$ integrate the transport reactions occurring at the PM and the VM, as well as metabolic reactions and cytosolic buffer capacity. Our data suggest that the observed changes may be due to H$^+$-coupled transport reactions. In Arabidopsis cells, AtCLCa is the major H$^+$-coupled NO$_3^-$ transporter in the VM (6, 31). Therefore, to test whether AtCLCa is responsible for the variations detected in the cytosol, we conducted comparative experiments between GCs from the wild type and from clca-3 knockout plants expressing ClpHensor (Figs. 3 and 4). We found that $[\text{NO}_3^-]_{\text{cyt}}$ reaches a steady-state value faster in clca-3 GCs than in the wild type when exposed to extracellular KNO$_3$ (Fig. 3). This proves that in vivo the vacuolar transporter AtCLCa buffers the $[\text{NO}_3^-]_{\text{cyt}}$, as expected from its function in accumulating $\text{NO}_3^-$ into the vacuole (6, 31). This finding may explain the defect of stomata opening reported earlier on isolated epidermis and dehydration test on whole rosettes (7). Gas exchange measurements showed that, on detached leaves, the application of

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KNO₃ induces an increase in the stomata conductance with a similar trend in both the wild type and clca-3 (SI Appendix, Fig. S10). Further, in the same experiments both genotypes reacted similarly to the application of ABA. These results seem to be in contrast with the observations made at the level of stomata in isolated epidermis from clca knockout (7) (Fig. 5). Such discrepancy is not unique to clca mutants as it was reported for other well-known how these mutants involved in stomata ABA signaling such as, for example, slac1, abi1, and abi2 (41). Nevertheless, all these mutants as clca display strong defects in tolerance to drought stress at the whole-rosette or whole-plant level. Notably, high concentrations (i.e., 50 μM) of ABA applied at the petiole of detached leaves are required to induce stomata closure in slac1 and abi mutants (41). In such conditions, other anion channels, like ALMT12/QUAC1, may bypass SLAC1 loss of function to allow stomata closure (26). 

At the subcellular level, the most impressive consequence of knocking out AtCLCa was on the pHcyt (Fig. 4). Indeed, in sharp contrast with wild-type GCs, no pH acidification could be detected in clca-3 GCs when [NO₃] increased. These unexpected findings reveal that AtCLCa solely accounts for the pH acidification detected in wild-type GCs. Moreover, we found that the absence of AtCLCa also perturbs pHcyt regulation during stomata opening after treatment with fuscocein (Fig. 5). The role of AtCLCa in the control of pHcyt is therefore not limited to situations involving massive changes of the concentration of its anionic substrate. Together, the results highlight a previously overlooked role of AtCLCa in pHcyt homeostasis. AtCLCa is not the only H⁺-coupled transport system operating in the PM and VM of GCs (Fig. 6). However, our results indicate that under the conditions tested, the transport activity of AtCLCa is predominant and high enough to overcome the pH buffering capacity of the cytosol. Therefore, the use of a biosensor like ClopHensor allowed us to detect in vivo the activity of an intracellular transporter, AtCLCa, and its impact of the intracellular ion homeostasis. 

The finding that a vacuolar transporter influences pHcyt homeostasis opens a perspective on the cellular functions of intracellular ion transporters. A potential role of H⁺-coupled transporters in the regulation of pHcyt may account for the unexpected resilience to drought stress at the whole-rosette or whole-plant level. Notably, high concentrations (i.e., 50 μM) of ABA applied at the petiole of detached leaves are required to induce stomata closure in slac1 and abi mutants (41). In such conditions, other anion channels, like ALMT12/QUAC1, may bypass SLAC1 loss of function to allow stomata closure (26).

Cytosolic pH Control, a Framework for CLC Functions. The results presented here relate to a specialized plant cell type, the GCs. The effect of AtCLCa on pHcyt may account for the unexpected defect in stomata closure observed in clca knockout plants, while its function in loading anions into the vacuole would rather lead to the prediction that it is solely involved in stomata opening (7). In this context, modification of pHcyt could be an important component of AtCLCa function, as pHcyt is an important parameter in cell signaling (58). The results obtained with fuscocein argue in favor of this hypothesis. The treatment with fuscocein was performed on GCs from mature plants, which allowed monitoring changes in stomatal aperture in parallel with pHcyt and [anion] variations. The misregulation of pHcyt in clca correlated with the defect in stomata opening. During the initial pHcyt increase that was not affected in clca, the rate of stomata opening was similar in the wild type and clca. In the following phase, the defect in pHcyt recovery in clca mutant paralleled a drop in the rate of stomata opening. Cytosolic pH modifications may modulate ion transport systems and enzymatic reactions to trigger stomata opening or closure. For example, the activity of vacuolar H⁺ ATPase (V-ATPase) is modified by changes of the pHcyt (59). Our findings may also be relevant in the broader context of other eukaryotic CLC exchangers. Indeed, the function of intracellular CLCs has been interpreted assuming that their only role was to regulate the lysosomal, endosomal, or vacuolar lumen conditions (2). However, the cellular functions of the lysosomal CLC-7 and endosomal CLC-5 remain unclear in mammal cells. CLC-7 was proposed to acidify the lysosomal lumen, but only modest and controversial effects were detected (14, 16). In the case of CLC-5, endosomes from knockout mice present impaired luminal acidification (33). Nonetheless, the connection between endosomal acidification and the severe defects caused by CLC-5 mutations in Dent’s disease is still unclear (2). Indeed, renal failure associated with some mutations in CLC-5 present impaired endocytosis in tubular cells, which is independent of endosomal acidification (33). Intriguingly, pHcyt is known to affect endocytosis (60, 61). The results we report here suggest that in eukaryotic cells, intracellular CLCs are part of the cytosolic pH balance machinery. These findings open a perspective on the function of these exchangers in eukaryotic cells and may provide a framework to understand the pathophysiological disorders caused by mutations in human CLC genes.

Methods
Wild-type Arabidopsis plants were Col-0 ecotype. The clca-3 knockout line corresponds to Gabi Kat GK-82AE03-02319. Images were acquired with a Leica SP8 upright CLSM. Image analysis was performed with ImageJ. Detailed description of the methods is available in SI Appendix.

Data Availability. All data presented in the paper are described in the text and SI Appendix. Biological materials are available from the corresponding author on request.

ACKNOWLEDGMENTS. This work was supported by LabEx Saclay Plant Sciences-SPS (ANR-10-LABX-0040-SPS) and by the ATIP-AVENIR-2018 program. P.C.-F. was supported by a postdoctoral grant from Fundación Alfonso Martín Escudero. This work has benefited from the facilities and the expertise of Imagerie-Gif microscopy platform, which is supported by France-BioImaging (ANR-INSB-04 “Investments for the future”) and by Saclay Plant Science (ANR-11 IDEX-0003-02). We thank M. Dauzat (Laboratoire d’Ecophysiologie des Plantes sous Stress Environnementaux) and N. Sibié (Institut for Integrative Biology of the Cell) for the help with experiments, Joni Frederick for reading the manuscript, D. Arosio (Consiglio Nazionale delle Ricerche) for providing plasmid with ClopHensor and advice, R. Le Bars (Imagerie-Gif) for the help with microscopy, and M. Bianchi (Institut for Integrative Biology of the Cell) and S. Filleur (Institut for Integrative Biology of the Cell) for discussion.

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