

Quantification of SnRK1.1 response through analysis of its intracellular distribution

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Key Message: SnRK1 response and cellular energy status are evaluated using a novel N/ER index, which reflects changes in the intracellular distribution of its catalytic subunit, SnRK1.1.

Abstract

Maintaining energy homeostasis is a major challenge for plants facing changes in growth conditions. The Sucrose non-Fermenting 1 (SNF1) Related Kinase 1 (SnRK1) complex is a central player in securing cell energy homeostasis. The α subunit of this complex, also known as SnRK1.1, is a protein kinase that plays a critical role in sensing energy status and coordinating metabolic reprogramming to counter any energy imbalance. The discovery of a dual and dynamic intracellular distribution of SnRK1.1 suggests that its activity and function might be regulated by spatiotemporal changes.

To investigate the link between the spatiotemporal localization of SnRK1.1 and SnRK1 response, we developed a protocol to quantify its intracellular distribution. We conceptualized and defined a new parameter, the N/ER index, which quantifies changes in distribution between nuclear and non-nuclear SnRK1.1 fractions. Using fluorescence confocal images acquired along the z-axis in plants expressing SnRK1.1–eGFP, and the open-source software Fiji/ImageJ, we calculated this parameter under control conditions and in plants treated with DCMU, a well-known trigger of SnRK1 response. These results showed that changes in SnRK1.1 intracellular localization constitute a major mechanistic step in the SnRK1-mediated response to restore energy homeostasis *in planta*. In addition, we establish the compatibility of our robust and simple method with a commercial software-based approach with different segmentation and quantification tools. Finally, our work demonstrates that N/ER index serves as a readout of SnRK1 response cell energy levels.

Key words: SnRK1.1, intracellular distribution, Fiji, energy homeostasis, DCMU

Introduction

Changes in growing conditions profoundly affect the growth and development of plants. The increasing frequency of unexpected climatic events negatively impacts on central plant processes like photosynthesis, ultimately affecting growth and development (Becklin, et al. 2021). To sustain energy homeostasis and buffer energy imbalances caused by non-optimal conditions, plants rely on several intracellular mechanisms (Xiao, et al. 2024). Among these, the Sucrose non-Fermenting 1 (SNF1) Related Kinase 1 (SnRK1) complex plays a central role (Wurzinger, et al. 2018; Peixoto and Baena-Gonzalez 2022). SnRK1 shares commonalities with its orthologs in yeast (SNF1) and in mammalian cells, the AMP-dependent protein kinase (AMPK) (Broeckx, et al. 2016; Emanuelle, et al. 2016). In response to decreases in cellular energy levels, SNF1/SnRK1/AMP kinase complex family orchestrates a shift toward catabolic reactions while suppressing energy-consuming anabolic processes, generating an energy surplus to withstand adverse growth conditions (Broeckx, et al. 2016). However, differences between the complexes of photosynthetic and non-photosynthetic organisms suggest plant-specific adaptations in bioenergetic regulation. For instance, SnRK1 is a heterotrimeric kinase complex that contains a $\beta\gamma$ subunit instead of the canonical γ subunit found in SNF1 and AMPK complexes (Emanuelle, et al. 2015; Emanuelle, et al. 2016). The presence of the plant-specific $\beta\gamma$ subunit in the plant holoenzyme promotes a distinct folding and regulation of the other α and β subunits. In the case of the catalytic α subunit, the spatial organization of its “activation loop” (or “T-loop”), a domain whose phosphorylation promotes kinase activity, makes it less accessible and more resistant to dephosphorylation than in non-photosynthetic counterparts (Emanuelle, et al. 2015). Additionally, the T-loop in SnRK1.1 lacks the regulatory conformational changes observed in AMPK and SNF1 upon binding of AMP (Shen, et al. 2009; Gowans, et al. 2013) or AMP and ADP (Xiao, et al. 2011), respectively (Oakhill, et al. 2012; Emanuelle, et al. 2015; Emanuelle, et al. 2016). Therefore, SnRK1.1 kinase is likely to be constitutively active, as its T-loop remains predominantly phosphorylated (Ramon, et al. 2019). Together, these peculiarities of the SnRK1.1 kinase suggest a unique regulatory mechanism for its activity complex reflecting the plant-specific lifestyle (Roustan, et al. 2016; Crepin and Rolland 2019; Peixoto and Baena-Gonzalez 2022).

The discovery of different intracellular SnRK1.1 fractions in plant cells has been crucial for studying the SnRK1-mediated signalling pathway from a spatiotemporal perspective (Gutierrez-Beltran and Crespo 2022). SnRK1 has been defined as a sensor or integrator of stress signals, and as a coordinator of the response, the latter function being related to the nuclear regulation of transcription (Baena-Gonzalez, et al. 2007). The nuclear fraction of SnRK1.1 has been linked to the phosphorylation and regulation of the activity of transcription factors and the concomitant regulation of gene expression (Ng, et al. 2013; Mair, et al. 2015; Muralidhara, et al. 2021; Peixoto, et al. 2021; Henninger, et al. 2022). The identification of a

second fraction of SnRK1.1 associated with the endoplasmic reticulum (ER) indicates a separation of function among different intracellular SnRK1.1 pools, suggesting that the ER might be a point of gauging and/or integration of low-energy signals (Jamsheer, et al. 2018; Blanco, et al. 2019; Crepin and Rolland 2019; Ramon, et al. 2019; Gutierrez-Beltran and Crespo 2022). In favour of this hypothesis, the ER has been identified as the colocalization site of SnRK1.1 with some of its interactors, including DUF581-FLZ family proteins (Jamsheer, et al. 2018; Jamsheer, et al. 2022), class II T6P synthase (TPS)-like proteins (Van Leene, et al. 2022), and members of the TOR complex (Nukarinen, et al. 2016). Furthermore, in leaf sections, changes in the SnRK1.1 fraction at the ER were triggered by different blockages of photosynthetic electron flow, which affects energy status (Blanco, et al. 2019). Depending on the final redox status of the electron transport chain, either overoxidized by DCMU or overreduced by DBMIB, SnRK1.1 fraction either delocalizes from the ER or rearranges into bright cytosolic puncta. These studies, plus the changes in the SnRK1.1-eGFP localization in mesophyll protoplasts expressing different β subunits of SnRK1 complex (Ramon, et al. 2019) and in root cells in response to ABA treatment (Belda-Palazon, et al. 2022) indicate a likely specific role of the different SnRK1.1 fractions (Blanco, et al. 2019; Gutierrez-Beltran and Crespo 2022). Although the underlying mechanism has not been fully elucidated, growing evidence suggests that SnRK1.1 activation is spatially and temporally regulated, an essential factor that must be considered when investigating mechanisms of energy homeostasis.

The participation of SnRK1 in cellular processes is currently determined by two methodological approaches based either on the analysis of the target genes or on the phosphorylation status of target proteins (Baena-Gonzalez, et al. 2007; Peixoto, et al. 2021; Van Leene, et al. 2022). In the first case, the quantification of the expression levels of SnRK1.1 target genes (e.g., *SENESCENCE-ASSOCIATED PROTEIN 5 (SEN5)*, *DARK INDUCIBLE 1 (DIN1/SEN1)*, and *6/ASPARAGINE SYNTHASE 1 (DIN6/ASN1)*, and *PROLINE DEHYDROGENASE 1 (PRODH)*) has been used as proxies of the SnRK1.1 activity (Baena-Gonzalez, et al. 2007; Pedrotti, et al. 2018; Peixoto, et al. 2021; Peixoto and Baena-Gonzalez 2022). Regarding the SnRK1 kinase activity, the identification of the SnRK1.1-dependent phosphoproteome or protein-protein interactions studies complemented by detection of target phosphopeptides have been used to verify SnRK1 activity (Cho, et al. 2016; Nukarinen, et al. 2016; Carianopol, et al. 2020; Van Leene, et al. 2022). Additionally, two methods to assess SnRK1 activity have also exploited its kinase activity *in planta* (Muralidhara, et al. 2021; Sanagi, et al. 2021; Avidan, et al. 2023; Safi, et al. 2023). Sanagi and co-workers generated Arabidopsis lines expressing a synthetic peptide derived from the rat Ser79 phosphorylation site of ACETYL COA CARBOXYLASE 1 (ACC), a conserved direct phosphorylation target of AMPK/SNF1/SnRK1.1 (Sanagi, et al. 2021). Relative SnRK1 activity is determined by the

quantification of immunoblot signals detected by antibodies anti-ACC pS79 compared to those of anti-HA or GFP, both domains included in the reporter construct. Different modifications of the technique have been used to determine the relative activity of the SnRK1 nuclear fraction by adding a localization sequence (NLS) to the AAC-peptide (Muralidhara, et al. 2021; Belda-Palazon, et al. 2022; Henninger, et al. 2022; Avidan, et al. 2023). In the second method, Safi and colleagues also express an optimized SnRK1.1 phosphorylation recognition motif (denominated AMPK substrate peptide (ASP)) tagged with GFP, but also fused to a homooligomerization coiled-coil sequence (HOTag) (Safi, et al. 2023). In this design (denominated ASP-SPARK), the phosphorylation of the ASP sequence promotes the oligomerization of the HOTag peptide, inducing a phase separation of this oligomeric reporter peptide. This process produces the appearance of large phase-separated ASP-SPARK condensates visualized as bright fluorescent puncta. Therefore, the quantification of the number of fluorescent puncta by confocal microscopy is the readout of SnRK1.1. kinase activity (Safi, et al. 2023; Persyn, et al. 2024). Although the readout ASP-SPARK provides information on SnRK1 at the cellular level, the phase separation principle underlying the technique partially obscures its precise intracellular localization. Additionally, the liquid-liquid phase separation kinetics may limit the ability to accurately determine the temporal dynamics of a SnRK1.1-mediated response.

In this work, we present a protocol to quantify the intracellular localization of SnRK1.1. Our protocol, based on the analysis of z-stack sets of images of cells expressing SnRK1.1-eGFP under a native promoter, provides an excellent tool to track changes in the intracellular distribution of SnRK1.1 fractions (hereinafter named SnRK1.1-OE) (Blanco et al, 2019). The readout of the method is an index between the nuclear and non-nuclear SnRK1.1 fractions, N/ER index. As proof-of-concept, we evaluated the SnRK1.1-mediated response to DCMU treatments via N/ER index as well as T-loop phosphorylation and protein kinase content. These results demonstrate that changes in SnRK1.1 intracellular localization constitute a key mechanistic step in the SnRK1-mediated response to restore energy homeostasis *in planta*. In addition, we confirm the compatibility of the method with a commercial software-based approach with different segmentation and quantification tools. Finally, our work demonstrates that N/ER index serves as a readout of both SnRK1.1 response and cell energy levels.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana plants were grown in a growth chamber under a 16 h light/8 h dark cycle, with an irradiance of 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and temperatures of 23° C/21 °C. *Arabidopsis* lines were initially selected on half-strength Murashige and Skoog (1/2 MS) medium

177 supplemented with 35 $\mu\text{g ml}^{-1}$ kanamycin for SnRK1.1–eGFP transgenic lines. Fifteen-day-
178 old plants were used for experiments.

179 Cell energy perturbation treatment

180 Cell energy perturbation experiments were conducted in transgenic *Arabidopsis* lines
181 expressing SnRK1.1-eGFP (SnRK1.1-OE) under a native promoter (Blanco et al., 2019). Two-
182 week-old plants were sprayed either with a mock solution (0.005% DMSO in water) or with 50
183 μM DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], each on separate sets of plants, and
184 imaged at their respective time points.

185 Fluorescence microscopy imaging

186 Images of pavement cells from leaves of *A. thaliana* SnRK1.1-OE lines were acquired at 0.25,
187 1, 2 and 5 hours post- treatment (Ctrl or DCMU) by Laser Scanning Confocal Microscopy
188 (LSCM) using a Zeiss LSM 880 microscope. Imaging was performed using a Plan-Apochromat
189 20x (NA = 0.8)Zeiss objective. Z-stack were acquired with a step size of 1 μm and a pinhole
190 diameter of 1 Airy Unit (AU). Samples were excited with a 488 nm laser and the emission was
191 detected with a photomultiplier set in integration mode, using 490-526 nm (eGFP) and 690-
192 710 nm (Chlorophyll autofluorescence) spectral filters. Analyses were conducted using at least
193 three biological replicates from independent experiments.

194 Image analysis

195 Z-stack images were analysed using the open-source software Fiji/ImageJ (Schindelin, et al.
196 2012) and IMARIS (Bitplane) software. In Fiji, images were processed using threshold and
197 Gaussian Blur filter tools to generate nuclei and ER masks. The average intensity fluorescence
198 measurements were obtained from the Measure tool of the ROI manager. Images were pre-
199 processed using ROF filters (Fiji) for IMARIS analyses. Contrast was calculated as $(I_r - I_b)/I_b$,
200 where I_r and I_b represent the mean intensity of the region of interest and the background,
201 respectively. Then, Nuclei and ER were segmented using the IMARIS automatic surface
202 rendering mode, selecting only those planes that included each region. This software was also
203 used to calculate the mean intensity of both structures for each image.

204 Western blot analysis

205 *Arabidopsis thaliana* transgenic and wild-type lines were used to obtain the total soluble
206 proteins. Briefly, 200 mg of leaves were ground in liquid nitrogen and homogenized in 4 ml of
207 extraction buffer (50 mM Tris–HCl, pH 7.5; 0.33 M sucrose; 5 mM EDTA; 150 mM NaCl, and
208 1X complete protease inhibitor cocktail from Roche). The total protein extract was obtained by
209 centrifugation at 10,000 g for 1 min with concentrations ~ 4 mg/ml as quantified using the
210 Sigma Bradford reactive, loading 40 μg per lane. Proteins were resolved by 12% (v/v) SDS-
211 PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% (w/v)
212 low-fat milk powder in TBS and then incubated with specific antibodies: anti-GFP-specific

(1:3000, Cell Signaling #2555), anti-SnRK1.1 (1:1000, Agrisera AS21 4581), and anti-T loop (1:1000, Cell Signaling #2535). The membranes were further incubated with anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) (1:2000, Cell Signaling #7075). Chemiluminescence detection was performed using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific), and the signal was visualized using an Amersham Imager 600.

Photosynthetic measurements

The effects of the DCMU treatments in Arabidopsis plants were measured in 2-week-old control (Col-0) and SnRK1.1-OE lines growing at 120 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$. Plants from both genotypes were randomly assigned to receive either the treatment (DCMU) or a mock solution (dilution of DCMU solvent, 0.005% DMSO, in water), by the same protocol as in energy perturbation treatments above. At specific time points (0, 0.25, 1, 2, or 5 h) PAM photosynthetic measurements and OJIP curves were obtained using FluorPen FP 110 and PhotosynQs MultispeQ PAM fluorometers. For maximum quantum yield of PSII (F_v/F_m) measurements, leaves were dark-adapted previously for at least 20 minutes to allow full reoxidation of the electron transport chain.

Statistical analysis

The N/ER index and flowering time differences between lines were detected using analysis of variance (ANOVA) followed by Tukey's HSD test (significance threshold $p < 0.05$), in InfoStat software (version 2020e) and its interface with R (Di Rienzo, et al. 2011). N/ER index was obtained using the average fluorescence of each fraction (denominated FL-Nuc-ROI and FL-ER-ROI for nuclear and ER-associated SnRK1.1 fractions, respectively) from z-stacks at each time point (0.25, 1, 2 and 5 h after mock or DCMU) in 3-4 independent experiments. Flowering time was obtained by averaging the number of days from germination until flower bud observation (0.5 cm) for each line ($n=17$). The number of rosette leaves was counted right after bud formation for each line ($n=17$). Linear regression of the N/ER indexes data obtained with the different software (Fiji/IMARIS) was calculated using InfoStat software and setting the y-intercept value to zero. Model significance level $p\text{-value} < 0.001$.

Results

SnRK1.1 is distributed in the whole volume of pavement cells in different intracellular fractions

Recently, our group and others have revealed that Arabidopsis SnRK1.1 is dually distributed between the surface of the endoplasmic reticulum (ER) and the nucleus (Jamsheer, et al. 2018; Blanco, et al. 2019; Ramon, et al. 2019; Gutierrez-Beltran and Crespo 2022). In these works, the distribution of SnRK1.1 was determined in different tissues and plant species by

the imaging of a fusion protein with a fluorescent tag (e.g., GFP and YFP). For instance, we used a fusion of a genomic fragment containing SnRK1.1 and its native promoter with eGFP at its C-terminal, including a linker of 15 amino acids between the last exon and the fluorescent tag to produce transgenic stable lines (SnRK1.1-OEs) (Blanco, et al. 2019). Moreover, we found that SnRK1.1 intracellular distribution was affected by changes in cell energy status produced by inhibition of photosynthesis (Blanco, et al. 2019).

To conclusively evaluate any change in SnRK1.1 intracellular localization, we developed a new protocol for the quantification of its distribution in stable transgenic SnRK1.1-OE Arabidopsis lines. First, we confirmed the expression of functional SnRK1.1-eGFP by different approaches (Fig. 1). Under long-day conditions, either in soil or in 0.5× MS medium, none of the SnRK1.1-OE lines (SnRK1.1-OE1 and SnRK1.1-OE2) exhibited clear phenotypic differences, compared to the control lines (Col-0), transgenic lines expressing eGFP targeted to the nucleus or *snrk1.1*^{-/-} (Fig. 1a). In the initial screening, SnRK1.1-eGFP fusion proteins were detected in leaf tissue of transgenic lines by western blot using commercial antibodies against eGFP (Fig. 1b). Considering that a fraction of SnRK1.1 is attached to the ER, which possesses different subdomains in pavement epidermal cells, we conducted imaging of leaf sections of SnRK1.1-OE plants along the z-axis, namely z-stacks. Indeed, the obtained focal planes in each z-stack showed different SnRK1.1 population ranging from those linked to the cortical ER at the abaxial periclinal cell surface to other fractions localized at the anticlinal ER domain. A set of images spanning from abaxial to adaxial leaf section side belonging to transgenic line SnRK1.1-OE2 is shown in Fig. 1c. Imaging experiments can be performed either using a single fluorescent channel corresponding to the fluorescent tag of the fusion protein or using multiple channels—including brightfield, an ER marker, or chlorophyll autofluorescence—as references to identify the different regions of the leaf pavement cells (Supplementary Fig. S1). In Fig. 1c, a 3D-rendered visualization of the SnRK1.1 intracellular distribution is shown in a 5-cell ROI of a leaf section, of fluorescence-tagged lines expressing SnRK1.1-eGFP. Beyond detecting SnRK1.1-eGFP by western blot (see also Fig. 3) and LSCM images, we assessed the functionality of the fusion protein via flowering time studies. SnRK1.1 overexpression has shown a delayed flowering (Baena-Gonzalez, et al. 2007; Williams, et al. 2014; Jeong, et al. 2015; Belda-Palazon, et al. 2020). Accordingly, the SnRK1.1-OE lines had a 3-day delay in flowering bud formation compared to Col-0 and *snrk1.1*^{-/-} lines, confirming the overexpression of an active SnRK1.1-eGFP fusion (Supplementary Table S1, Supplementary Fig. S2a).

After establishing the imaging methodology and plant model, we analysed multiple z-stacks to develop a quantification protocol of SnRK1.1 intracellular distribution. We chose the comparison of the median fluorescence intensity of the SnRK1.1 fraction at the anticlinal ER

domain and in the nucleus as a quantification strategy (Fig. 2b). This approach assures consistency in the fluorescence intensity along the SnRK1.1 ER fractions of different z-stacks. The selection of the SnRK1.1 fractions associated with the anticlinal ER domain is representative of the average intensity of the whole non-nuclear fraction of SnRK1.1. Furthermore, the selected fractions have a more even distribution of the SnRK1.1-eGFP signal, avoiding the inclusion of fluorescent foci that are more common in cortical sections. This prevents quantification processes from being skewed by small areas with saturated fluorescence intensities. Additionally, at the cellular level, the SnRK1.1 fraction in the anticlinal ER domain adjacent to the nucleus is more likely to participate directly in nuclear-directed SnRK1-mediated responses than fractions in other ER regions. This provides a more relevant basis for assessing the regulation of nuclear gene expression by SnRK1.1.

Quantification of SnRK1.1 fractions and generation of a N/ER index as the concept of cell energy status determinations.

Visualizing SnRK1.1 intracellular distribution in z-stacks of leaf sections from SnRK1.1-OE plants and selecting focal planes corresponding to SnRK1.1 fractions at the anticlinal ER domain and in the nucleus constitutes the first stage of our quantification protocol (Fig. 2a). Using this criterion and measuring strategy, we identified and selected the positions of the focal planes for each SnRK1.1 fraction (ER-associated, hereinafter referred to as ER-SnRK1.1; and nuclear, Nuc-SnRK1.1) in the z-stack of images acquired from sections of a plant leaf (Fig. 2b-I). Any difficulty in identifying the respective focal planes can be overcome using the corresponding image from the brightfield channel and/or from the ER-marker channels of the acquired stacks as a reference. The following steps in the pipeline involved the use of Fiji/ImageJ, an open-source imaging analysis software, and yielded the mean fluorescence intensity values of representative images for each of the SnRK1.1 fractions (Fig. 2b) (Schindelin, et al. 2012). The z-stack files were opened with Fiji and split into their different channels (use the command *Image > Color > Split Channels*), and sub-stacks corresponding to the SnRK1.1 channel were processed to define masks for segmenting the fluorescence signal of the ER-SnRK1.1 and Nuc-SnRK1.1 (Fig. 2b-I). Three to four z-slices (step size=1 μ m) in the z-axis were selected from each stack, corresponding to ER-SnRK1.1 and Nuc-SnRK1.1 (using the command *Duplicate > Range* and choosing z-slice range for different SnRK1.1 fraction) (Fig. 2b-II). Each set of z-slices was then processed to generate a single representative image for the SnRK1.1 fraction by applying the Z-projection function with the average intensity tool (set of commands *Image > Stack > Z-projection > Average Intensity*). Each resulting “projection” image was duplicated for further analysis. These newly generated z-projection images were named “ER-SnRK1.1-proj” and “Nuc-SnRK1.1-proj” (Fig. 2b-II), and served to determine the mean fluorescence intensity for each SnRK1.1 fraction. From these images, two binary images were generated to segment each SnRK1.1 fraction (sequences of

commands *Image > Type > 8-bit > Adjust > Threshold > Apply > Process > Filters > Gaussian Blur > Binary > Make Binary > Convert to Mask*). Alternatively, ER-marker brightfield images can be used to refine the generated masks, which can improve the accuracy of SnRK1.1 intensity quantification in the “ER-SnRK1.1-proj” and “Nuc-SnRK1.1-proj” images.

Next, a region of interest (ROI) was defined for each projection image using the corresponding masks, which yielded the average fluorescence of the ER- and Nuc-SnRK1.1 fractions. The ROI was generated using the Fiji Wand tool within the ROI manager by clicking on each mask. If the mask creation process yielded multiple ROIs, they were combined using the command *ROI management > More > OR (combine) > Add*. The mean fluorescence intensity within the ROIs of ER-SnRK1.1 and Nuc-SnRK1.1 was then measured (*ROI management > Select ROIs > Measure > Mean Intensity*), resulting in two values: “FL-ER-ROI” and “FL-Nuc-ROI” (Fig. 2b-III). Finally, the ratio between “FL-Nuc-ROI” and “FL-ER-ROI” was calculated, yielding a numerical value referred to as “N/ER index” (Fig. 2c). This N/ER index quantifies the relative distribution of SnRK1.1 between the ER-associated and nuclear fractions within the cell volume, providing insight into its intracellular localization and dynamic behaviour.

Proof-of-Concept: Evaluating the Effect of DCMU on SnRK1.1 Intracellular Distribution

Changes in the distribution of SnRK1.1 have been observed during developmental processes and in response to various environmental cues (Zhai, et al. 2017; Blanco, et al. 2019; Han, et al. 2020; Belda-Palazon, et al. 2022; Shi, et al. 2024). To validate our protocol, we evaluated the previously reported effect of photosynthetic electron transport blockage on the intracellular distribution of SnRK1.1 (Blanco, et al. 2019). Our previous results showed that changes in SnRK1.1 localization primarily occurred in vascular tissues after 1 h of treatment with DCMU, an inhibitor that binds to the quinone binding site B (Q_B) of photosystem II and blocks electron flow from PSII to plastoquinone (Takahashi et al. 2010) (Supplementary Fig. S3a and Table S2).

Using 2-week-old SnRK1.1-OE plants, we imaged leaf sections from plants sprayed with 50 µM DCMU or mock-treated over time (Fig. 3). In pavement cells expressing SnRK1.1-eGFP, we observed the same behaviour of the fusion protein previously reported in vascular tissue (Fig. 3). An apparent increase in the Nuc-SnRK1.1 fraction was observed at the expense of the ER-SnRK1.1 fraction, as visualized in 3D reconstructions of the analysed sections (Fig. 3a). For each time point, we applied our protocol to at least three z-stack series of various individuals, across three independent experiments (Fig. 3b, Supplementary Fig. S4). The visual trend observed in LSCM images was confirmed through quantification, which showed a statistically significant increase in the N/ER index between 1 and 5 h after treatment

(i.e., from 2.38 ± 0.38 before treatment to 2.76 ± 0.65 and 3.21 ± 0.21 , after 1 and 5 h after treatment, respectively, $p < 0.01$) (Fig. 3a-b, see also Bottom table Fig. 4).

To gain further insight into this response, we evaluated endogenous and overexpressed SnRK1.1 protein level by western blot on imaged sections (Fig. 3c). No changes in expression levels were observed in either endogenous SnRK1.1 or SnRK1.1-eGFP content between DCMU- and mock-treated leaf sections, nor over time within each treatment (Fig. 3c upper blot). This was further confirmed using anti-GFP antibodies, which specifically recognize SnRK1.1-eGFP (Fig. 3c middle blot). The absence of high-mobility signals in the 20–35 kDa range confirmed the integrity of the fusion protein, indicating that the fluorescence signal identified as Nuc-SnRK1.1 (Fig. 2b) accurately corresponds to SnRK1.1-eGFP, rather than to a potentially cleaved eGFP tag relocalized to the nucleus. Furthermore, we evaluated whether phosphorylation of the T-loop was associated with these changes in SnRK1.1 intracellular distribution. As observed for SnRK1.1 protein level, no differences in T-loop phosphorylation were detected in either endogenous SnRK1.1 or SnRK1.1-eGFP in response to DCMU treatment (Fig. 3c lower blot). Moreover, the degree of phosphorylation of the T-loop was stable in nuclear and non-nuclear fractions of the leaf tissue (Supplementary Fig. S6). Finally, using various photosynthetic measurements, including OJIP curves, we confirmed that DCMU treatment resulted in a complete and sustained blockage of photosynthetic electron transport throughout the experiment (Fig. 3d, Supplementary Fig. S3 and S5, and Table S2). Analysis of the first and last time points of the treatment showed the abolishment of the JIP phases, indicating an effective blockage of the electron flow downstream of Q_A in Photosystem II (PSII) (upper and lower panels in Fig. 3d; full timeframe in Supp. Fig. S5) (Tóth et al. 2005).

The drop in q_L values to ~ 0.2 , and the complete loss of the $Y(II)$ parameter confirm the absence of photosynthetic transport in DCMU-sprayed leaves throughout the treatment period, regardless of SnRK1.1-eGFP overexpression (Supplementary Fig. S3). The DCMU treatment caused a small decrease in the maximum quantum yield (F_v/F_m), which might indicate a perturbation in the funneling of absorbed energy into PSII, but not permanent damage.

These results demonstrate the changes in SnRK1.1. distribution, quantified by the N/ER index, were triggered by a total interruption of photosynthetic electron flow and the resulting decrease in photosynthetic energy production over the 5-hour treatment period.

Compatibility of our quantification protocol for SnRK1.1 intracellular distribution with other software tools

To assess the compatibility of our quantification protocol, which includes various segmentation and quantification steps, with alternative analysis tools, we analyzed the DCMU treatment data using IMARIS (Bitplane, Belfast, Northern Ireland, UK). This software is widely used for

advanced multidimensional image visualization, segmentation, and quantitative analysis (Fig. 4). Specifically, we used the same z-stack sets as in Fig. 3b from mock- and DCMU-treated plants over time (0.25-5 h), to calculate the N/ER index based on the fluorescent intensity in different subcellular fractions of SnRK1.1-eGFP, by segmentation with IMARIS. Before segmentation, the images were pre-processed with a ROF filter (Fiji), which reduces noise while preserving edges and enhancing image quality. Next, nuclei and ER were segmented using IMARIS's automatic surface rendering mode, applying a user-defined intensity threshold to identify structures with a contrast value above ~3, and a size threshold of 8 μ m for nuclei segmentation, set to exclude small, non-biologically relevant elements. Only those planes corresponding to each region of interest were selected for analysis (Fig. 4a). Finally, the software was used to calculate the mean intensity of both structures in each segmented 3D image. As in Fiji-based analysis, we determined SnRK1.1 changes in its intracellular distribution over time by calculating the N/ER index for mock (Ctrl) and DCMU-treated plants (Fig. 4b). Consistent with our Fiji procedure (Fig. 3b), we observed an enrichment of SnRK1.1 in the nucleus after 2 and 5 h of DCMU-treated plants (Fig. 4b).

The scatter-plot for the pair of N/ER indexes obtained by our protocol and by using IMARIS showed a linear correlation (Fig. 4c, Supplementary Table S3a-b), with highly significant correlation coefficients of $r^2=0.98$ ($p < 0.0001$, mock) and $r^2=0.99$ ($p < 0.0001$, DCMU), respectively. The high correlation was also observed in the averaged values per time-point (Fig. 4c, Supplementary Table S3a-b). To estimate the robustness and precision for both protocols, we calculated the coefficient of variation throughout the complete set of N/ER indexes ($n=38$ and $n=45$, for mock and DCMU, respectively). Coefficients of variation (CVs) for Fiji measurements were between $12.6\% \pm 6.1\%$ and $17.0\% \pm 8.4\%$ for mock- and DCMU-treated plants, respectively. For IMARIS, CVs were between $15.9\% \pm 9.6\%$ and $19.3\% \pm 8.1\%$ for mock- and DCMU treated-plants, respectively. CVs remained moderate across different experiments (mock and DCMU), with a slight overperformance for Fiji measurements. Overall, this evaluation confirmed that the quantification protocol is robust, reliable, and compatible with different segmentation and quantification approaches.

Discussion

Significant efforts have been made to understand how SnRK1 restores energy homeostasis since the initial characterization of Arabidopsis SnRK1.1 mutant lines (Baena-Gonzalez, et al. 2007). Beyond identifying its target genes, phosphorylation consensus motifs, target proteins, and interactors, the recent discovery of distinct intracellular fractions of SnRK1.1 has provided new insights into its activity (Jamsheer, et al. 2018; Blanco, et al. 2019). Furthermore, changes in SnRK1.1 intracellular localization in response to the blockage of photosynthesis by DCMU have also contributed to deciphering its activation mechanisms (Fig. 3) (Blanco, et al. 2019;

Ramon, et al. 2019). Currently, there is consensus that the activity of SnRK1.1 signalling pathway is linked with its spatiotemporal fingerprint, a characteristic also observed in its opisthokont counterparts (Zong, et al. 2019; Gutierrez-Beltran and Crespo 2022). This emerging model is built upon the idea of a spatial separation of roles for SnRK1.1: sensing energy imbalance at the ER and mediating metabolic reprogramming by regulating gene expression in the nucleus to restore energy homeostasis (Wurzinger, et al. 2018; Blanco, et al. 2019; Crepin and Rolland 2019; Ramon, et al. 2019; Gutierrez-Beltran and Crespo 2022; Peixoto and Baena-Gonzalez 2022). Due to its central role, this form of "retrograde signalling" also involves intracellular crosstalk with other regulatory pathways, notably its co-regulation with the TOR complex (Nukarinen, et al. 2016; Belda-Palazon, et al. 2022; Jamsheer, et al. 2022).

Based on this model, we designed a protocol to assess SnRK1 response by quantifying SnRK1.1 intracellular distribution. Starting from z-stacks of fluorescent images obtained from stable SnRK1.1-eGFP lines, the distribution of GFP signal between the nucleus (Nuc-SnRK1.1) and the ER (ER-SnRK1.1) is quantified using Fiji, which serves as a tool for segmenting and quantifying each fraction separately. The results are expressed through a new parameter, the N/ER index, which normalizes expression differences between cells. The choice of Fiji in this protocol offers a simple, free, and open-source solution for both segmentation and quantification, and it is suitable for automation through Fiji macros. In terms of segmentation, the effectiveness of Fiji is enhanced by two factors: (i) the selection of specific plant tissue to image, and (ii) the choice of z-planes within the cells. Leaf tissue is an optimal model for evaluating the impact of growth conditions on plant cell energy status and SnRK1.1 distribution (i). In particular, the pavement cells selected in our study exhibit photosynthetic performance that is sensitive to external cues (Dopp et al. 2023). Additionally, due to their distinctive morphology, pavement cells facilitate the segmentation of the different compartments where SnRK1.1 is associated, namely nucleus and ER surface (ii) (Fig. 2a). For Nuc-SnRK1.1, selecting focal planes at the most abaxial position of the z-stacks, just before the position of spongy parenchyma in the z-axis, enables easier segmentation of nuclei by discrete ROIs, thereby improving the quantification of this fractions. As previously mentioned, the focal planes spanning the SnRK1.1 fraction associated with the anticlinal ER provide a representative intensity value for the entire ER-associated pool. The validity of this segmentation and quantification strategy using Fiji was confirmed by the similar N/ER index results obtained with IMARIS, used here as an alternative segmentation software for quantifying the Nuc-SnRK1.1 and ER-SnRK1.1 intensity (Fig. 4). Moreover, experiments where the plants were previously treated with DCMU indicate no changes in the levels of SnRK1.1 or the SnRK1.1-eGFP fusion protein (Fig. 3c), supporting the conclusion that

changes in the N/ER index primarily reflect an increase in the fluorescence signal from the Nuc-SnRK1.1 fraction, at the expense of the ER-SnRK1.1 fraction (Fig. 3 and 4, DCMU treatment). These results are consistent with previous observations in vascular tissue treated with DCMU and ABA-induced response in roots (Blanco, et al. 2019; Belda-Palazon, et al. 2022). Interestingly, these changes in SnRK1.1 intracellular distribution also occurred independently of T-loop phosphorylation (Fig. 3c). Structural predictions and *in planta* analyses have also shown that T-loop phosphorylation is stable and may play a limited role in modulating SnRK1.1-mediated response (Emanuelle, et al. 2016; Ramon, et al. 2019). To date, significant changes in T-loop phosphorylation have only been observed within 30 minutes following submergence treatment (Cho, et al. 2016). Aside the SnRK1.1 activation due to T-loop phosphorylation, our results cannot ruled out additional mechanisms that might regulate T-loop phosphorylation status or SnRK1.1 activity, which can be studied by kinase activity based methods (see below alternative methods for determining SnRK1.1 kinase activity). However, our results support the hypothesis that changes in SnRK1.1 localization are key mechanistic steps in the current working model of SnRK1-mediated responses to restores energy homeostasis.

One advantage of studying SnRK1.1 intracellular distribution via the N/ER index is the compatibility of this approach with different image segmentation and quantification methods. Both Fiji—the initial method used to establish the protocol—and IMARIS yielded similar N/ER indexes when evaluating SnRK1.1 response to photosynthesis blockage (Figs. 3 and 4), with a single statistical difference observed at 1 h of DCMU treatment. A possible explanation is that the automatic thresholding routine used with IMARIS produces the inclusion of a broader population of ER-associated SnRK1.1, including a fraction located near cortical adaxial regions. These areas are characterized by fewer and smaller chloroplasts with lower photosynthetic activity (Mackenzie and Mullineaux 2022). Consequently, a higher ER-SnRK1.1 signal may result from reduced photosynthetic inhibition compared to the anticlinal ER regions adjacent to highly active mesophyll chloroplasts. A similar pattern was previously observed in the dynamic behaviour of SnRK1.1 in vascular tissues (Blanco, et al. 2019). Despite these minor differences, DCMU treatment effectively blocked photosynthetic electron transport and altered cellular energy status, validating the protocol. The loss of photosynthetic activity was confirmed by OJIP transients and PAM measurements (Fig. 3; Supplementary Figs. S3 and S5). DCMU induced complete inhibition of photosynthetic electron transport in illuminated leaves of both control and SnRK1.1-OE, as shown by significant reductions in q_L and $Y(II)$ (Supplementary Fig. S5). The disappearance of characteristic shoulders and plateaus at photosynthesis induction in the OJIP curves further confirmed this effect (Supplementary Fig. S3). A mild and transient reduction in F_v/F_m was also observed, indicating

no lasting damage and ruling out any permanent oxidative stress caused by the DCMU treatment. Importantly, under both DCMU- and mock-treatments, the robustness and reproducibility of our quantification protocol were confirmed by the strong correlation between the results of Fiji and IMARIS. This was evidenced by the high r^2 value (~ 0.98) and highly significant $p < 0.0001$), confirming the reliability of the N/ER index as a comparative metric for SnRK1.1 localization.

The homogeneity, stability, and robustness of N/ER index values across segmentation methods, treatments, and time points highlight its strength as a reliable parameter to quantify SnRK1.1 intracellular distribution. Under control conditions (long-day 16:8 h, 100–120 $\mu\text{mol m}^{-2} \text{ s}^{-1}$, ZT3–ZT8), the N/ER index value (~ 2.25) can serve as a proxy for the energy status of non-stressed plant cells in leaves. Accordingly, deviations from this index value can be used as readouts for assessing the quality of growth conditions and their effect on cell energy status. Needless to say, more extensive applications of the N/ER index using different growth conditions are needed to confirm it. Independent of this, our approach can be readily integrated with other existing tools to study SnRK1 response. *A priori*, its compatibility with phase separation-based visualization of kinase activity using ASP-SPARK constructs may not be straightforward (Safi, et al. 2023). However, the ASP-SPARK reporter method could be applied sequentially to determine the kinetics of general SnRK1.1 activity in response to a stimulus, followed by N/ER index quantification to map the spatial localization of downstream phosphorylation events. In parallel, ACC phosphorylation motif-based reporter methods (Sanagi, et al. 2021), in particular newer versions incorporating an NLS and eGFP for nuclear SnRK1.1 activity quantification (Muralidhara, et al. 2021; Belda-Palazon, et al. 2022; Avidan, et al. 2023), share spatiotemporal compatibility with our N/ER index protocol. When used together, these tools can help dissect the sequence of molecular events regulating SnRK1.1 activation and deactivation during energy homeostasis recovery. These studies can be useful to address three key questions regarding SnRK1.1 activity: *i*) Is SnRK1.1 activity fully independent of T-loop phosphorylation? *ii*), is T-loop phosphorylation required for SnRK1.1 intracellular localization? and *iii*) are changes in localization part of the mechanism that deactivates SnRK1.1 or *vice versa*? So far, current evidence supports a positive answer to (*i*), but the roles of T-loop phosphorylation in localization and regulation of SnRK1.1 activity (*ii* and *iii*) remain unknown. An interesting application of these complementary methods would be the design of an ACC-based reporter driven by SnRK1.1 regulatory sequences and targeted to the ER surface using N-terminal tags such as the N-myristoylation site of SnRK1 β 1 and β 2 or an ER membrane marker sequence (McFarlane, et al. 2017; Ramon, et al. 2019; Wang, et al. 2019).

In summary, the N/ER index offers a simple, rapid, and robust method to quantify SnRK1.1 intracellular dynamics over time. Beyond SnRK1.1, our quantification protocol can be adapted to study any signalling protein whose activity involves changes in intracellular distribution. Accordingly, it serves as a useful tool to refine signalling protein interactomes by assigning spatial and temporal context to protein–protein interactions. Developing specific indices for different intracellular compartments—and tracking these values over time for a given protein—represents a promising strategy to establish a spatiotemporal hierarchy that enables the differentiation between direct and indirect interactors. Among the most compelling candidates for this type of analysis is the TOR complex, which antagonizes SnRK1 activity and may be particularly well suited for such spatiotemporal studies.

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Conflict of interest

No conflict of interest declared.

Author contribution statement

NEB conceived the project and experiments. NEB and CB conceptualized the N/ER index. DL created plant lines. CB grew the plants, performed microscopy and DCMU experiments, designed the protocol with Fiji and performed the N/ER index acquisition. FJH helped CB with Fiji protocol. CD and VL performed the N/ER index measurements using IMARIS. JB obtained and analyzed the photosynthetic measurements. ADL performed Western Blot. CMF helped obtain Flowering data, performed statistics, and helped with the linear regression analysis. JD helped in the discussion. NEB, CB, CMF, CD and VL wrote the manuscript. All authors read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary Table S1. Flowering time and number of rosette leaf data.

Supplementary Table S2. Data of Photosynthetic parameters results of mock and DCMU treatments.

Supplementary Table S3A-B. Linear Regression data of the N/ER index obtained by Fiji and IMARIS software.

Supplementary Figure S1. Single fluorescence channel.

Supplementary Figure S2. SnRK1.1-OE exhibits a delay in flowering time.

Supplementary Figure S3. Validation of DCMU effectiveness through photosynthetic measurements.

Supplementary Figure S4. N/ER INDEX experiments resulting from the application of a new protocol.

Supplementary Figure S5. OJIP curves for DCMU response.

Supplementary Figure S6. Evaluation of T-loop phosphorylation status in subcellular fractions of DCMU-treated plants.

741 **Data availability**

Data is included in Supplementary information. Any data is also available upon request to blanco@cefobi-conicet.gov.ar. **Supplementary table 1- Flowering time in SnRK1.1 lines**

Line	Individual	Days to flower	Number of rosette leaves
<i>snrk1.1^{-/-}</i>	1	21	15
<i>snrk1.1^{-/-}</i>	2	20	14
<i>snrk1.1^{-/-}</i>	3	17	12
<i>snrk1.1^{-/-}</i>	4	17	14
<i>snrk1.1^{-/-}</i>	5	18	13
<i>snrk1.1^{-/-}</i>	6	20	15
<i>snrk1.1^{-/-}</i>	7	20	13
<i>snrk1.1^{-/-}</i>	8	17	11
<i>snrk1.1^{-/-}</i>	9	19	14
<i>snrk1.1^{-/-}</i>	10	17	11
<i>snrk1.1^{-/-}</i>	11	18	11
<i>snrk1.1^{-/-}</i>	12	18	13
<i>snrk1.1^{-/-}</i>	13	20	13
<i>snrk1.1^{-/-}</i>	14	17	12
<i>snrk1.1^{-/-}</i>	15	18	13
<i>snrk1.1^{-/-}</i>	16	19	13
<i>snrk1.1^{-/-}</i>	17	18	13
Col-0	1	19	13
Col-0	2	17	13
Col-0	3	17	11
Col-0	4	18	12

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Col-0	5	19	14
Col-0	6	16	13
Col-0	7	19	13
Col-0	8	18	13
Col-0	9	20	13
Col-0	10	18	12
Col-0	11	22	14
Col-0	12	18	14
Col-0	13	20	13
Col-0	14	19	14
Col-0	15	20	14
Col-0	16	21	14
Col-0	17	19	13
SnRK1.1- <i>OE1</i>	2	22	14
SnRK1.1- <i>OE1</i>	3	22	16
SnRK1.1- <i>OE1</i>	4	23	16
SnRK1.1- <i>OE1</i>	5	20	16
SnRK1.1- <i>OE1</i>	6	20	14
SnRK1.1- <i>OE1</i>	7	19	14
SnRK1.1- <i>OE1</i>	8	21	15
SnRK1.1- <i>OE1</i>	9	21	14
SnRK1.1- <i>OE1</i>	10	21	13
SnRK1.1- <i>OE1</i>	11	21	15
SnRK1.1- <i>OE1</i>	12	20	12
SnRK1.1- <i>OE1</i>	13	19	10
SnRK1.1- <i>OE1</i>	14	21	15

SnRK1.1- <i>OE1</i>	15	20	15
SnRK1.1- <i>OE1</i>	16	20	14
SnRK1.1- <i>OE1</i>	17	21	16
SnRK1.1- <i>OE2</i>	1	21	18
SnRK1.1- <i>OE2</i>	2	21	18
SnRK1.1- <i>OE2</i>	3	21	17
SnRK1.1- <i>OE2</i>	4	19	14
SnRK1.1- <i>OE2</i>	5	19	15
SnRK1.1- <i>OE2</i>	6	17	13
SnRK1.1- <i>OE2</i>	7	22	18
SnRK1.1- <i>OE2</i>	8	22	18
SnRK1.1- <i>OE2</i>	9	22	19
SnRK1.1- <i>OE2</i>	10	17	12
SnRK1.1- <i>OE2</i>	11	23	18
SnRK1.1- <i>OE2</i>	12	20	16
SnRK1.1- <i>OE2</i>	13	28	23
SnRK1.1- <i>OE2</i>	14	21	17
SnRK1.1- <i>OE2</i>	15	21	19
SnRK1.1- <i>OE2</i>	16	18	13
SnRK1.1- <i>OE2</i>	17	21	17

		<i>snrk1.1</i> ^{-/-}	Col-0	SnRK1.1- <i>OE1</i>	SnRK1.1- <i>OE2</i>
Days to flower	Means ± SD	18.47±1.33	18.82±1.51	20.69±1.08	20.76±2.56
Number of rosette leaves	Means ± SD	12.94±1.25	13.12±0.86	14.31±1.62	16.76±2.72

Supplementary table 1- Flowering time in SnRK1.1 lines

Genotype	Treatment	Treatment time (h)	Y(II)	Y(NO)	Y(NPQ)	qL	Fv/Fm
SnRK1.1- <i>OE</i>	Control	0.00	0.413 ± 0.040	0.120 ± 0.011	0.467 ± 0.050	0.708 ± 0.028	0.842 ± 0.005
		0.25	0.437 ± 0.037	0.137 ± 0.014	0.426 ± 0.050	0.657 ± 0.023	0.810 ± 0.046
		1.00	0.424 ± 0.032	0.124 ± 0.010	0.453 ± 0.041	0.703 ± 0.015	0.818 ± 0.026
		2.00	0.407 ± 0.034	0.113 ± 0.010	0.480 ± 0.043	0.737 ± 0.030	0.812 ± 0.022
		5.00	0.457 ± 0.021	0.134 ± 0.005	0.409 ± 0.025	0.701 ± 0.021	0.823 ± 0.016
Col-0		0.00	0.403 ± 0.061	0.118 ± 0.017	0.478 ± 0.077	0.698 ± 0.036	0.840 ± 0.009
		0.25	0.417 ± 0.035	0.125 ± 0.011	0.458 ± 0.047	0.685 ± 0.022	0.821 ± 0.024
		1.00	0.469 ± 0.033	0.137 ± 0.009	0.394 ± 0.041	0.704 ± 0.016	0.808 ± 0.043
		2.00	0.459 ± 0.054	0.128 ± 0.016	0.412 ± 0.069	0.735 ± 0.022	0.797 ± 0.025
		5.00	0.445 ± 0.040	0.127 ± 0.010	0.428 ± 0.050	0.719 ± 0.028	0.835 ± 0.007
SnRK1.1- <i>OE</i>	DCMU	0.00					
		0.25	0.035 ± 0.029	0.039 ± 0.009	0.926 ± 0.038	0.162 ± 0.123	0.682 ± 0.035
		1.00	0.018 ± 0.007	0.041 ± 0.005	0.942 ± 0.005	0.094 ± 0.045	0.657 ± 0.015
		2.00	0.032 ± 0.005	0.038 ± 0.003	0.930 ± 0.007	0.173 ± 0.026	0.617 ± 0.019
		5.00	0.044 ± 0.008	0.030 ± 0.002	0.927 ± 0.007	0.306 ± 0.064	0.541 ± 0.037
Col-0		0.00					
		0.25	0.035 ± 0.041	0.041 ± 0.011	0.924 ± 0.051	0.138 ± 0.160	0.682 ± 0.019
		1.00	0.022 ± 0.006	0.038 ± 0.006	0.941 ± 0.005	0.124 ± 0.051	0.617 ± 0.040
		2.00	0.032 ± 0.005	0.037 ± 0.007	0.930 ± 0.006	0.185 ± 0.058	0.621 ± 0.027
		5.00	0.038 ± 0.005	0.034 ± 0.002	0.928 ± 0.004	0.232 ± 0.035	0.564 ± 0.027

Supplementary Table S2. Data of Photosynthetic parameters results

747 a)

Treatment	N/ER-IMARIS	N/ER-Fiji	Exp 1	Time
Ctrl	1.90	1.97	1	0.25
Ctrl	2.54	2.66	1	0.25
Ctrl	2.20	2.16	1	0.25
Ctrl	2.45	2.73	1	0.25
Ctrl	1.81	1.83	1	1.00
Ctrl	2.60	1.97	1	1.00
Ctrl	2.39	2.26	1	1.00
Ctrl	2.05	1.91	1	1.00
Ctrl	3.41	2.70	1	1.00
Ctrl	1.41	1.93	1	2.00
Ctrl	2.17	2.21	1	2.00
Ctrl	2.22	2.56	1	2.00
Ctrl	2.41	2.30	1	2.00
Ctrl	1.74	1.84	1	5.00
Ctrl	1.65	1.79	1	5.00
Ctrl	1.55	1.87	1	5.00
Ctrl	1.69	2.25	1	5.00
DCMU	2.07	2.26	1	1.00
DCMU	2.13	2.51	1	1.00
DCMU	2.39	2.23	1	1.00
DCMU	2.43	2.70	1	1.00
DCMU	2.27	2.37	1	1.00
DCMU	2.98	3.28	1	1.00
DCMU	3.58	3.99	1	1.00
DCMU	1.52	1.83	1	0.25
DCMU	2.17	2.66	1	0.25
DCMU	2.58	2.95	1	0.25
DCMU	2.91	3.25	1	0.25
DCMU	2.38	2.90	1	2.00
DCMU	2.50	2.64	1	2.00
DCMU	2.64	3.59	1	2.00
DCMU	3.74	3.70	1	2.00
DCMU	2.80	2.41	1	2.00
DCMU	2.24	2.91	1	5.00
DCMU	3.42	3.22	1	5.00
DCMU	3.05	3.38	1	5.00
DCMU	3.16	3.34	1	5.00

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750

751 b)

Linear Regression data								
Exp	Treatment	Variable	N	R ²	Adj R ²	PMSE	AIC	BIC
1	Ctrl	N/ER-IMARIS	17	0.98	0.98	0.13	14.72	16.38
Regression coefficients								
Coef	Est.	S.E.	LL(95%)	UL(95%)	T	p-value	Mallows' Cp	VIF
N/ER-Fiji	0.98	0.04	0.90	1.06	26.01	<0.0001	675.64	1.00
Analysis of variance table (Partial SS)								
S.V.	SS	df	MS	F	p-value			
Model	79.06	1	79.06	676.64	<0.0001			
N/ER-Fiji	79.06	1	79.06	676.64	<0.0001			
Error	1.87	16	0.12					
Total	80.93	17						
Exp	Treatment	Variable	N	R ²	Adj R ²	PMSE	AIC	BIC
1	DCMU	N/ER-IMARIS	20	0.99	0.99	0.10	10.94	12.93
Regression coefficients								
Coef	Est.	S.E.	LL(95%)	UL(95%)	T	p-value	Mallows' Cp	VIF
N/ER-Fiji	0.91	0.02	0.86	0.96	40.72	<0.0001	1657.06	1.00
Analysis of variance table (Partial SS)								
S.V.	SS	df	MS	F	p-value			
Model	144.58	1	144.58	1658.06	<0.0001			
N/ER-Fiji	144.58	1	144.58	1658.06	<0.0001			
Error	1.66	19	0.09					
Total	146.24	20						

Supplementary Table S3 a) Raw data for Linear regression. b) Linear regression

Figure 1. SnRK1.1 is distributed throughout the entire plant cell volume. Set of lines used in this study and their control lines (*snrk1.1*^{-/-} is the KO line *snrk1α1-3* (GABI_579E09), Belda-Palazon et al., 2020) growing under long-day conditions, either in soil or in 0.5×MS medium (a). Western blot of total protein extract of leaf sections from different SnRK1.1-OE plants using anti-GFP antibodies during the initial screening of positive transformants, with image of loading control using Ponceau S (b). Images obtained by LSCM of leaf sections from *A. thaliana* plants overexpressing SnRK1.1-eGFP (c). A series of images from a z-stack, spanning from the adaxial to the abaxial side: (1) SnRK1.1-eGFP signal in the cortical ER (Cort-ER), (2) SnRK1.1-eGFP signal in the anticlinal ER (Ant-ER), (3) SnRK1.1-eGFP signal in the nucleus (Nuc), and (4) SnRK1.1-eGFP signal in the abaxial-side ER and chloroplasts in the bottom spongy tissue layer. A 3D reconstruction of SnRK1.1 distribution in a leaf pavement cell based on the z-stack images in a plant expressing SnRK1.1-eGFP (Blanco et al., 2019). Chloroplasts are shown in blue, SnRK1.1 in green, and the z-axis/total volume in a white-lined box. Images were captured by Zeiss LSM 880 microscope and processed using Zeiss ZEN 3.10 Lite software. Scale bars: 10 cm (a), 10 μm (c).

Figure 2. Pipeline of the protocol designed in Fiji/ImageJ to quantify SnRK1.1 intracellular distribution. The protocol is divided into three main stages: Imaging (a), quantification of segmented fluorescence in different SnRK1.1 fractions (b), and parameterization of SnRK1.1 distribution using the N/ER Index (c). A detailed description of each step is provided in the text.

Figure 3. Study of the SnRK1.1-mediated response to the blockage of photosynthesis. An integrative analysis of SnRK1.1 intracellular dynamics was performed using the N/ER index quantification protocol to understand its response to photosynthesis blockage. A 3D reconstruction of SnRK1.1-eGFP distribution changes in SnRK1.1-OE plants treated with DCMU for 2 h (a). The N/ER index was calculated from z-stacks of DCMU- (dark grey box plots) and mock-treated (Ctrl, light grey box plots) plants (b). In parallel, endogenous and alien fusion protein levels were assessed by Western blot using anti-SnRK1.1 (1:1000 Agrisera AS21 4581), phospho-AMPKα (T172) (1:1000 Cell Signaling #2535), and GFP (1:3000 Cell Signaling #2555) antibodies (c). The signal corresponding to the exogenous fusion protein is indicated with an open-headed arrow, while the endogenous SnRK1.1 signal is marked with a solid-headed arrow. The effectiveness and stability of DCMU treatment over time were confirmed by chlorophyll a fluorescence transient in OJIP curves (d). The DCMU treatment abolished the JIP phases of the OJIP curves (solid symbols, DCMU curves) compared to mock-treated plants (open symbols, Ctrl curves) throughout the evaluated period, as represented by measurements taken at 15 minutes (upper graph) and 5 h (lower graph) indicating a total stop in photosynthetic energy production. Asterisks (*) indicate statistically significant differences ($p < 0.05$), determined by one-way ANOVA followed by Tukey HSD test.

Figure 4. Quantification of N/ER index protocol using an alternative segmentation software. Representative 3D image reconstruction and segmentation obtained from SnRK1.1-eGFP signal in Nuclei and ER using a commercial 3D specialized microscopy analysis software (IMARIS). Scale bar = 20 μ m. (a). Analysis of SnRK1.1 distribution over time (0 to 5 h) using the N/ER index calculated with IMARIS software, for mock-treated (Ctrl, light-grey) or 50 μ M DCMU-treated plants (dark-grey). Statistically significant differences between treatments were detected using one-way ANOVA followed by Tukey HSD test. Asterisks (*) indicate significant differences (p -value < 0.05 (b). Scatter-plot for pairs of N/ER indexes obtained with Fiji or IMARIS (same set of confocal images used in the calculation of the Nuclear and ER sections of SnRK1.1), for mock-treated (Ctrl, light-grey) or DCMU-treated plants (dark-grey). Linear regression was used to estimate the correlation coefficient for each experiment (R^2). Table of average N/ER index obtained with Fiji and IMARIS (Means $\pm$ SD, n=4-7) (c).

Supplementary Figure S1. Single fluorescence channel. 3D rendering reconstruction of Z-stack images in individual fluorescence channels: SnRK1.1-eGFP (a), ER-RFP (b), and chloroplast auto-fluorescence (c), along with the brightfield image (d). A merged 3D reconstruction combining GFP, ER-RFP, and chloroplast autofluorescence is shown in (e). SnRK1.1 distribution could be analysed using the SnRK1.1-eGFP channel. ER-RFP channel aid to generate an ER mask for SnRK1.1-ER quantification. Scale bar: 20 μ m.

Supplementary Figure S2. SnRK1.1-OE exhibits a delay in flowering time. (a) Evaluation of flowering time in SnRK1.1 lines: *snrk1.1^{-/-}* (SnRK1.1 KO lines), Col-0 (wild-type), and SnRK1.1-OE lines (SnRK1.1-eGFP fusion lines, #1 and #2). Results show that SnRK1.1-OE lines exhibit a significant delay in flowering time (days) compared to Col-0. The *snrk1.1^{-/-}* line behaves similarly to wild-type plants. (b) Analysis of the number of rosette leaves revealed no significant differences between SnRK1.1-OE1, Col-0, and *snrk1.1^{-/-}* lines, except for SnRK1.1-OE2, which showed a significant variation. (c) Representative image illustrating differences in flowering time. Sample size (n) = 17. Different letters indicate statistically significant differences using analysis of variance (ANOVA) followed by Tukey's HSD test (significance threshold $p < 0.05$). Scale bar: 10 cm.

Supplementary Figure S3. Validation of DCMU effectiveness through photosynthetic measurements. Two-week-old wild-type plants (Col-0) and SnRK1.1-eGFP overexpressing (SnRK1.1-OE) plants were treated with DCMU or water (Ctrl). (a) Scheme of the effect of DCMU over the Electron Transport Chain. Electron flow is schemed as a red line. DCMU blocks the electron transport from quinone binding site A (Q_a) to site B (Q_b) in Photosystem II (PSII). PAM measurements of chlorophyll fluorescence were obtained to calculate (b) fraction of open PSII reaction centers, (c) PSII quantum yield under illumination and (d) maximum quantum yield of PSII in dark-adapted leaves.

Supplementary Figure S4. N/ER INDEX experiments resulting from the application of a new protocol. Independent experiments (Experiments 2 and 3) were conducted to quantify SnRK1.1 intracellular distribution under control conditions (Ctrl) and following treatment with 50 μ M DCMU over different time points (0.25, 1, 2, and 5 h). Both graphs illustrate that up to 1 h post-treatment, the N/ER INDEX increased significantly compared to the control in all time frames. Asterisks (*) indicate statistically significant differences ($p < 0.05$), determined by one-way ANOVA followed by Tukey HSD test.

Supplementary Figure S5. OJIP curves for DCMU response. Two-week-old Col-0 and SnRK1.1-OE (SnRK1.1-eGFP expressing lines) plants were exposed to 50 μ M DCMU (dark line) or Ctrl conditions with water (grey line). The same patterns are observed in both Col-0 (a) and SnRK1.1-OE (b) lines under both DCMU and mock treatments. Under DCMU treatment, OJIP curves show a saturated J peak, reflecting the complete reduction of the quinone binding site A in Photosystem II and maximal blockage of downstream electron flow.

Supplementary Figure S6. Evaluation of T-loop phosphorylation status in subcellular fractions of DCMU-treated plants. Subcellular fractionation and determination of SnRK1.1-eGFP in total, nuclear (Nuc), and non-nuclear (Non-nuc) soluble protein fractions in response to DCMU treatments. Different subcellular fractions were obtained from plants treated with DCMU (DCMU) or mock (Ctrl) following a protocol adapted from Blanco et al., 2019. SnRK1.1-eGFP and the T-loop phosphorylation status were assessed in the different fractions by western blot using the same antibodies as in Figure 3. Black arrows indicate the signal corresponding to endogenous SnRK1.1, while SnRK1.1-eGFP is indicated with white arrowheads. Loading controls are shown with Ponceau S-stained membranes. Ten percent of total proteins and Non-nuc fractions were loaded in comparison with the total of the Nuc fraction for analysis.

Fig1

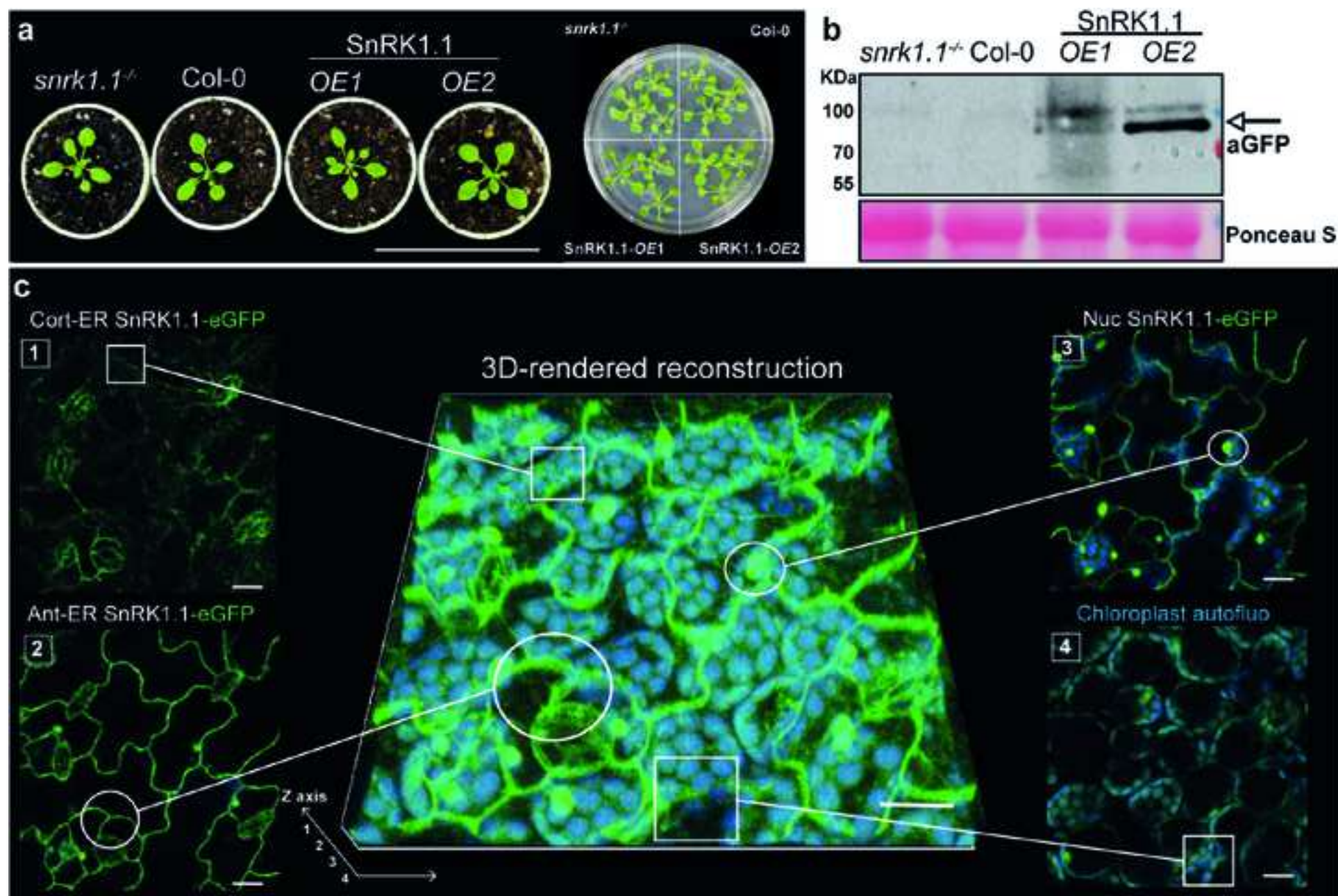


Fig2

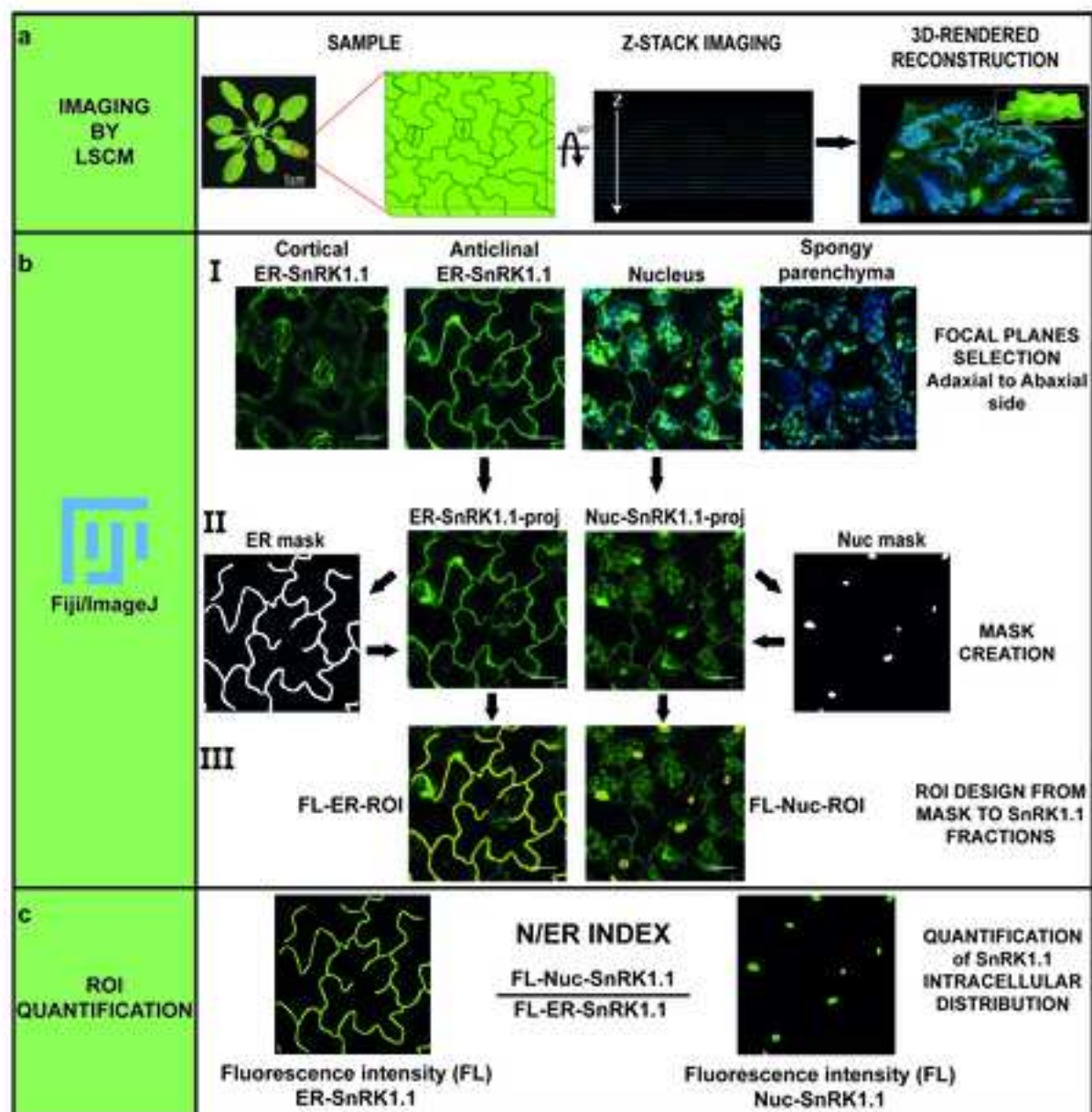


Fig3

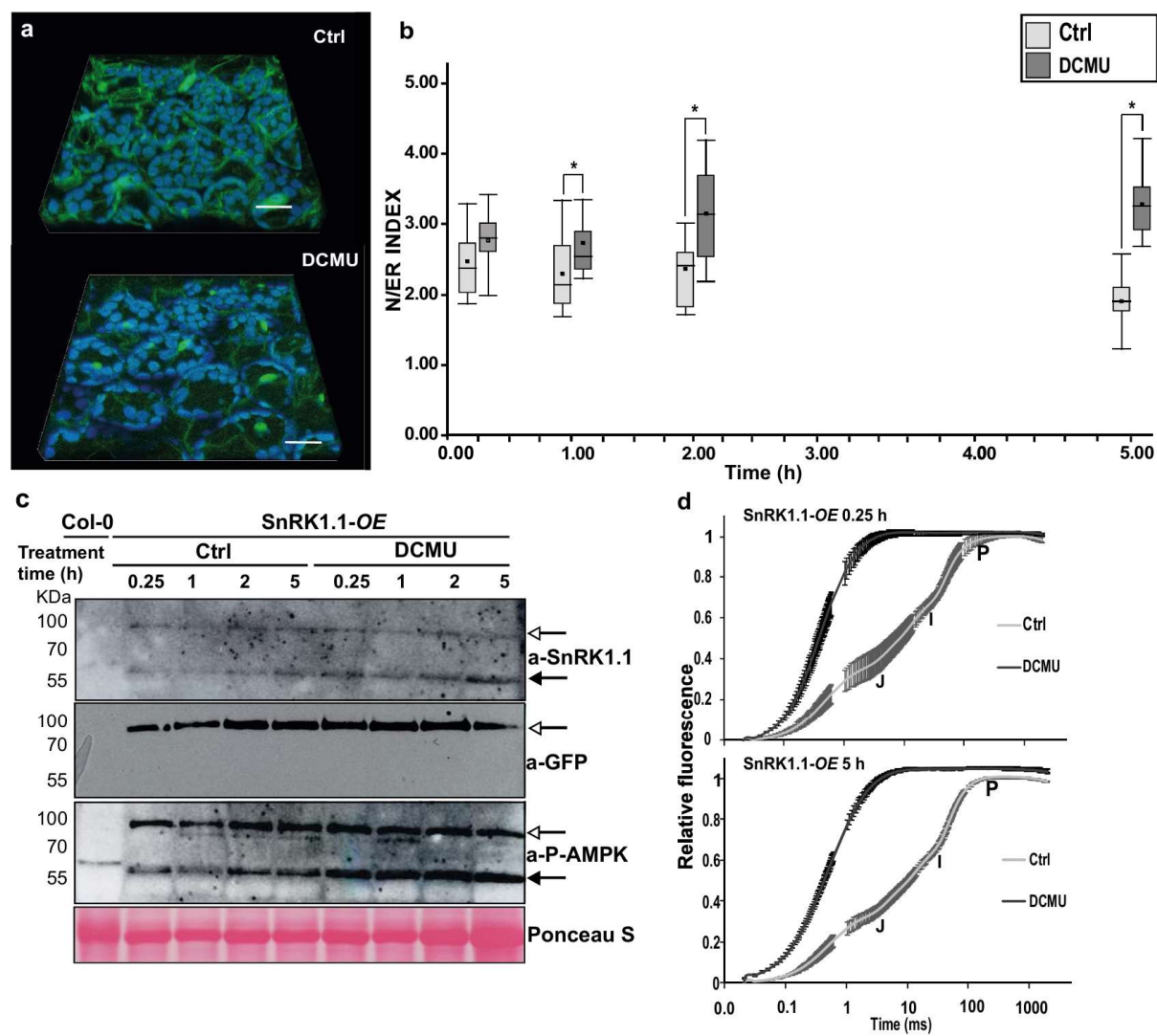


Fig4

