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# Going cresyl for plant cell imaging

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ABSTRACT

The advent of fluorescent probes and the characterization of their photochemical properties in the past years allowed significant advances in the studies of spatiotemporal cellular processes within complex and crowded systems. Dyes are indeed extremely useful tools for the visualization of cellular and subcellular structures present in living cells, as well as to study their dynamic and molecular composition or physiological changes. There are some areas of plant cell biology that have been more challenging to explore due to the physiology and organization of certain endomembrane compartments. In this study we characterize the labeling properties of cresyl violet as quick and inexpensive imaging agent for tracking endosomes, vacuole compartments which are usually very differentiated and categorized as more acidic as well as acidic plant compartments. Its photobleaching, labelling and cytotoxic properties are compared with other well-known and currently most used synthetic and molecular probes.

## 1. Introduction

The intracellular pH of plant cells is finely tuned to facilitate a series of metabolic reactions, ensuring protein stability, ion channel/transporter activity, cell compartmentalization, and endomembrane trafficking. All these processes are vital for plant growth and development and for their fast adaptation in challenging conditions, it is therefore important to follow and study all the cell compartments that are involved in the maintaining of the cellular pH homeostasis. Plant cells are characterized by compartments that are more acidic than others such as vacuoles (lytic and storage vacuoles) and endosomes (various and heterogeneous populations of small compartments) or the apoplast. These cellular compartments morphology in terms of size and number are cell and developmental stage specific, for instance the studies related to endosomomes characterization have been particularly puzzling. Numerous methods exist for labeling endosomes and vacuoles on account of the accessibility of fluorescent probes. Commercially available chemical dyes result easy to use and the current methodologies allow to obtain results in a really short time, conversely, the approach of using genetically encoded markers relying on lumen-targeted proteins or membranes, could be more time-consuming, because longer is the time required to introduce these markers in the plant of interest. Among the most frequently employed fluorescent probes, the FM (Fei-Mao) dyes such as FM4-64, FM5-95 and FM1-43 are usually used to identify endosomes and stain the vacuolar membranes of plant cells [1–3,18]. FM-dyes enter cells through the endocytic vesicles produced from the plasma membrane thanks to their amphiphilic nature, which causes them to adhere to the outside leaflet of a bilayer [2,3]. FM-dyes are styryl-based dyes, which are undeniably advantageous for a variety of applications, although, their chemical properties have been shown to affect membranes fluidity [16].

The FM dyes can induce transitory processes that endure until a new steady state is achieved in response to abrupt changes in plasmamembrane (PM) composition [8,9]. The fleeting fluidity of PM is caused by the steric competition existing between FM dyes and PM proteins. Numerous receptor functions and mechanotransduction channels are also inhibited by FM dyes (for a review, see [6,7]. Although FM dyes are highly effective at tracing endocytosis in vivo, they are not capable of revealing the pH conditions of the cell compartments. Additionally, FM dyes do not label all plant endocytic pathways, only providing limited information on the compartments' presence. For instance, Jelinkova et al. [9,8] found no evidence of co-localization between FM-labeled endosomes and FM-induced auxin transporter PIN1(PIN-formed)-GFP patch.

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Through our research and extensive testing of fluorophores with high fluorescence intensity and photostability, we sought a probe specifically suited for characterizing these plant cell compartments. We identified cresyl violet, a traditional histological marker primarily used for animal cells, particularly neurons. Notably, cresyl violet had not been previously tested or employed for plant cell studies [11,14,21]. Cresyl violet is approximately half of the size and the molecular weight of FM4-64. By causing little disturbance to the endomembrane architecture of plant cells, cresyl violet would thereby become a practical probe for evaluating the spatiality and dynamics of acidic compartments such as endosome or vacuolar compartments. The use of this inexpensive synthetic fluorescent dye, can be advantageous for its efficient labeling properties, providing an indication of the pH range in distinct locations within plant cells (e.g., evaluate the acidic milieu and environment of endosomes and vacuoles).

Synthetic fluorescent dyes are generally limited in their application due to several challenges. These include issues related to compartmental specificity of labeling, penetration difficulties, particularly in plants, and most importantly, undesirable pleiotropic side effects on cellular processes. Cresyl violet, on the other hand, does not exhibit these problems. In our study, we demonstrated its remarkable performance by showing its ability to detect various endocytic populations and vacuole compartments in both *Arabidopsis thaliana* and *Nicotiana tabacum*.

The use of the commercially available chemical dyes that speed up the investigation on genetically unexplored plant species is often necessary in an era when scientists are investigating the utility of a vast array of plants that not necessarily are model plants.

## 2. Results and discussion

### 2.1. Characterization of cresyl violet fluorescence in plant cells

The cresyl violet has been so far only employed in animal and yeast cells [14] to monitor lysosome compartments. Working as an acidotropic substance, this dye has propensity to accumulate in the acidic compartments from where it originates. The scope of our work was to test the ability cresyl violet to penetrate and label plant cell compartments that have been difficult to study and characterize on the bases of their acidic characteristics. To do so, the performance and properties of cresyl violet in plant cells was first investigated focusing on its fluorescence spectral properties. We examined the spectra emission of cresyl violet in *A. thaliana* and *N. tabacum* plant tissue, using the numerous confocal laser lines at our disposal (Fig. 1A, B, C). These investigations were conducted both in root and leaf epidermal cells determining the emission spectra produced by each laser line: 458, 476, 488, 496, 514, and 543 nm. During these analyses we identified that the most effective excitation line that can be used in plant cells was 543 nm, indeed evaluating the emission values, this line resulted as the best compromise in terms of fluorescence intensity emitted by the cresyl violet and the detected chlorophyll autofluorescence (see Fig. 1A, B, C). This aspect becomes relevant in experiment in plant cells that couple cresyl violet labelling and the chloroplasts autofluorescence detection. Chlorophyll autofluorescence from chloroplasts can indeed interfere with analysis. Therefore, using a dye with the same excitation wavelength as chlorophyll can be problematic, leading to non-specific labeling. Therefore,

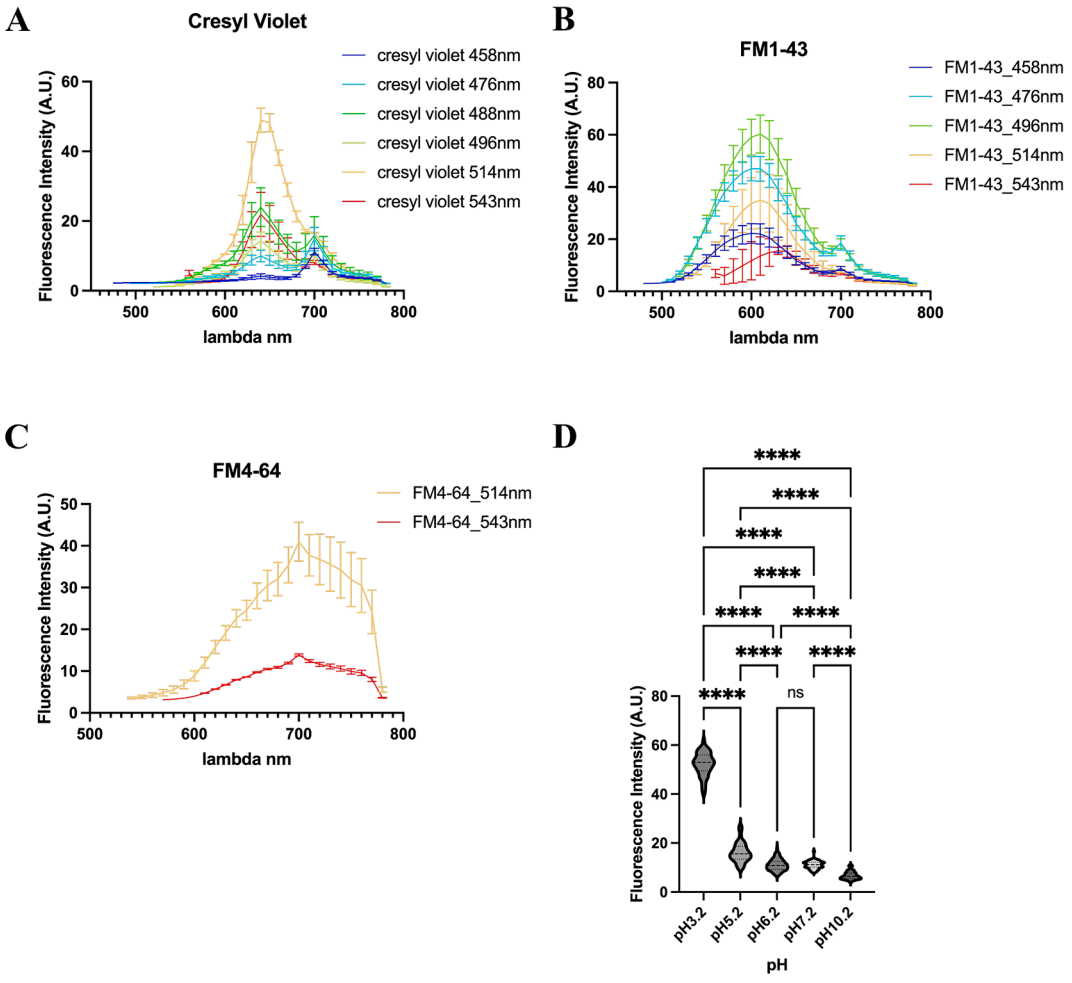


**Fig. 1.** Panel A, B, C. Cresyl violet (A) fluorescence emission spectrum characterization in comparison to the emission spectra of FM1-43 (B) and FM4-64 (C) when distinct excitation wavelengths are utilized. Panel D. Profile of the fluorescence of 50 μM cresyl violet in aqueous solutions at different pH values (3.2; 5.2; 7.2; 10.2).

this is an important aspect to consider in the studies on plant cells especially when leave samples are imaged. Furthermore, observing the emission produced at the different excitation wavelength tested, it is possible to notice that for cresyl violet the spectra is narrower compared to FM1-43 and FM4-64 (Fig. 1A, B, C). This is clearly an advantage when performing multiple labeling experiments with different fluorophores simultaneously. Identifying the exact fluorescence emission range detectable in plant tissue allowed us to conclude that cresyl violet could be easily used in colocalization experiments with most common fluorescent markers like CFP, GFP, and YFP obtaining a very distinct and specific signal. The following step consisted in using the identified 543 nm laser line to analyze the correlation between cresyl violet fluorescence intensity and the pH. We evaluated the cresyl violet emission in solutions at various pH levels: 3.2; 5.2; 7.2 and 10.2. As expected, the fluorescence intensity of cresyl violet showed higher values at the lowest pH values, and vice versa, a decrease in fluorescence intensity was observed at higher pH values (Fig. 1D). These data suggest that cresyl violet could be used as a fast and practical probe for the study and characterization of acidic compartments in plant cells; It has been found to be important not only to label the acidic compartments but also to measure, even if not precisely, the pH range of each of the labeled organelles. All the fluorescence properties of cresyl violet were tested in vivo in plant tissue, both root and leaf epidermal cells were used for confocal microscopy analysis.

### 2.2. Cresyl violet affinity for acidic endosomes and vacuoles

Once characterized the fluorescence spectra and tested its ability to mark and distinguish acidic compartments in vivo, the next target was to determine cresyl violet's usefulness and performance in the specificity of the labelling acidic environments, therefore, its distribution in diverse types of cells was monitored. The plant epidermal cells of root or leaf (Arabidopsis plants) were incubated for 5 min with 1 μM cresyl violet.

Upon the addition of cresyl violet, a number of different small organelles scattered throughout the cytoplasm were quickly intensely stained, then the staining progressively reached what appeared to be the vacuolar membranes. The punctate structures observed mapping the distribution of the dye resembled those that were seen when mapping the endocytic pathway through the use of other dyes such as FM1-43 or FM4-64 [3,19].

In order to pinpoint the organelles within plant cells that had accumulated cresyl violet we next performed colocalization analyses though confocal microscopy. For this purpose, we used FM1-43 as fast fluorescent labeling dyes to be added in combination with cresyl violet. The spectra emission of FM1-43 in plant tissue was first analyzed using the various confocal laser lines that were available in our confocal setup, this approach gave us the possibility to determine the best excitation laser line and emission spectra to be used for the co-labeling with cresyl violet avoiding fluorescence bleed-through, a phenomena occurring when fluorescence from a neighboring channel leaks in the channel of interest (Fig. 1A, B, C). Once we performed colocalization imaging and analysis between FM1-43 and cresyl violet a significant degree of colocalization with the structures of endosomes and vacuoles was obtained (Fig. 2). Therefore, being able to understand that cresyl violet preferentially label these organelles. To confirm this result, the stably transformed line of *A. thaliana* expressing the vacuolar fluorescent markers (VAC-YFP) was treated with either either a short or long (overnight) chase with cresyl violet and then observed at confocal to ensure that the marker made it to the final compartments after passing through the intermediate endocytic steps.

Endosomes and vacuoles can be targeted with this colorant by a straightforward adsorption or infiltration procedure, which is applicable to any type of plant, making this dye suitable to different plant model.

### 2.3. Cresyl violet performance in mapping different endosomes populations in plant leaf and root cells

According to recent studies that have demonstrated endosome heterogeneity, these organelles pH can vary significantly, even inside a single cell [12,17]. It was therefore crucial to determine if cresyl violet accumulated preferentially in certain endosome populations or whether it did so indiscriminately. The distribution of cresyl violet compared with several endosome fluorescent markers in both leaf and root epidermal cells was investigated. The endocytic markers SYP61(Syntaxin of Plants 61), RABF2a (Ras Analog in Brain), and RABF2b were utilized [15,19]. Time-course imaging analyses were conducted on leaf

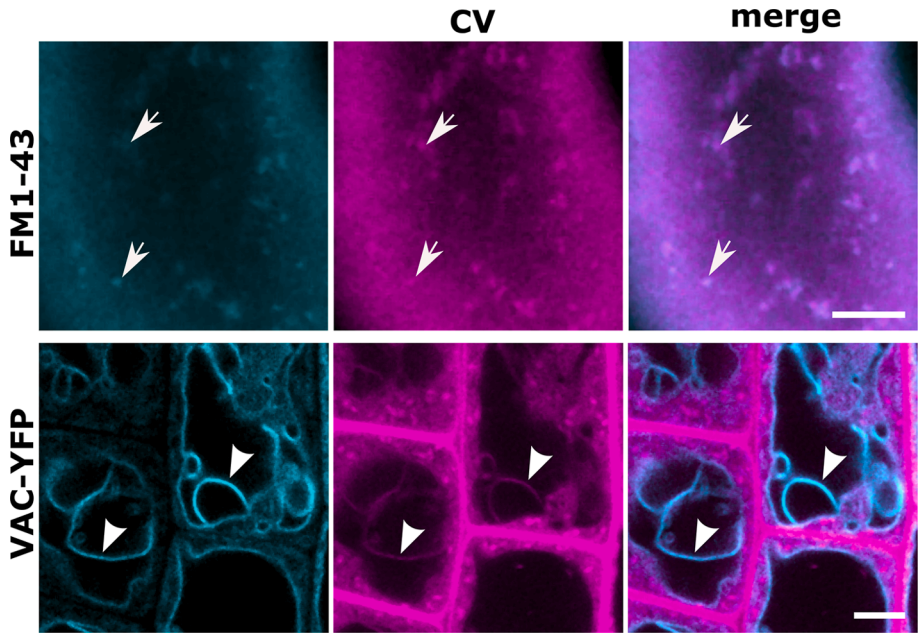


**Fig. 2.** Subcellular distribution of the endocytic tracer FM1-43 and cresyl violet in leaf epidermal cell of *Arabidopsis thaliana* (top panel). Confocal images (bottom panel) showing an association of cresyl violet with a vacuolar marker VACYFP. White arrowhead and arrow, indicate vacuole and endosome respectively. Scale bar, 5 μm.

and root epidermal sections that were labeled with the fluorescent tagged proteins CFP-SYP61, CFP-RabF2a and CFP-RabF2b in combination with the cresyl violet (Figs. 3 and 4. Late endosomes/MVBs (Multivesicular bodies) are marked by RabF2a; the EE (early endosomes) and LE (late endosomes) are both marked by RaF2b [4,10,19,20]. We detected, by means of dual-color live-cell imaging, that the populations of endosomes labelled by CFP-SYP61 or by the two Rab-fusion proteins exhibited a significant degree of association with the cresyl violet-labelled network (Figs. 3 and 4). However, and interestingly, none of these proteins labelled the entire populations extensively. According to the presented data, a significant proportion of the endosome populations that are observed when RabF2a and RabF2b are utilized, become persistently coupled with the endosome populations that are labeled with cresyl violet. In conclusion, using cresyl violet we were able to entirely map each endosome bodies, while the fluorescent markers used (SYP61, RABF2a and RABF2b) were only labeling a portion of the endomembrane compartments because each of them specifically localizes in a definite endocytic subdomain.

From these observations emerges that the cresyl violet is extremely useful for preliminary localization studies of uncharacterized genes predicted to be at endosome level. The localization analysis would be in this case facilitated because the cresyl violet would mark all the endosomes population simplifying the localization analysis. The use of specific genetic marker, in this case, will have the disadvantage to take longer due to the need to establish stable lines, and the results may only show a partial overlap, leading to ambiguity in gene localization.

### 2.4. Cresyl violet-labelling for endosomal trafficking and auxin efflux studies in plant roots

To further test the cresyl violet labelling performance in root and to analyze endosomal trafficking and the auxin efflux mechanisms, Arabidopsis seedlings stably expressing PIN1PIN1–GFP or PIN2PIN2–GFP [5,22] were treated with cresyl violet and the marker internalization across the plasma membrane was followed. As indicated by the punctate GFP signal, a significant increase in the internal pool of both fusion proteins was seen following a 5-minute treatment with cresyl violet. Unlike previous observations with the most common FM dyes, where FM-labeled endosomes displayed incomplete colocalization with PIN1PIN1-GFP or PIN2PIN2-GFP patches, cresyl violet internalization labeled endosomes that completely colocalized with these patches (Fig. 5).

### 2.5. Cresyl violet photostability in plant cells

In order to ascertain the photostability of cresyl violet, a steady-state fluorescence intensity measurement experiment was conducted. Cells that were labeled with cresyl violet were analyzed conducting time-lapse imaging acquisition at confocal. As obtained with the tests done utilizing endomembrane fluorescent markers, the stimulation laser power was maintained at a very low level to replicate more realistic settings. As shown in Fig. 6A, the photostability of cresyl violet and FM1-43 were compared. Following a two-minute imaging session utilizing identical laser line with consistent power settings, the photostability of the fluorescent proteins can be directly assessed by observing the temporal variation (increase or decrease) of the fluorescence intensity. It was observed that cresyl violet underwent very minimal photobleaching (about 9 percent), in contrast to the FM1-43 signal which was diminished by more than 26 percent (Fig. 6A). Our findings indicate that cresyl violet exhibits a significantly reduced propensity for photobleaching, this makes of this marker an ideal dye for long acquisitions, like it could be the case of a timelapse allowing in this way also the tracking of specific cell compartments over a certain period of time.

### 2.6. Cresyl violet cytotoxic properties evaluation

To establish whether or not the cresyl violet could chemically interfere with cell endomembrane homeostasis affecting the endomembrane compartments structure or morphology, we performed an experiment of colocalization where this dye was observed in seedlings stably expressing first the Golgi markers STGFP (sialyl transferase) and GAYK (Golgi apparatus), then the endoplasmic reticulum marker (ERYK) [13]. Upon the dye application, both the endomembrane compartments, endoplasmic reticulum (ER) and the Golgi retained their intact organellar structure, even after a prolonged exposure to the dye, demonstrating that the cresyl violet has no cytotoxic effects (Fig. 6B, C, D). Furthermore, differently from FM4-64 which after a prolonged exposure labels also the Golgi compartments [3], the cresyl violet only partially stains the Golgi stacks (Fig. 6D high magnification) probably locating in the small region of overlapping between the (Trans Golgi

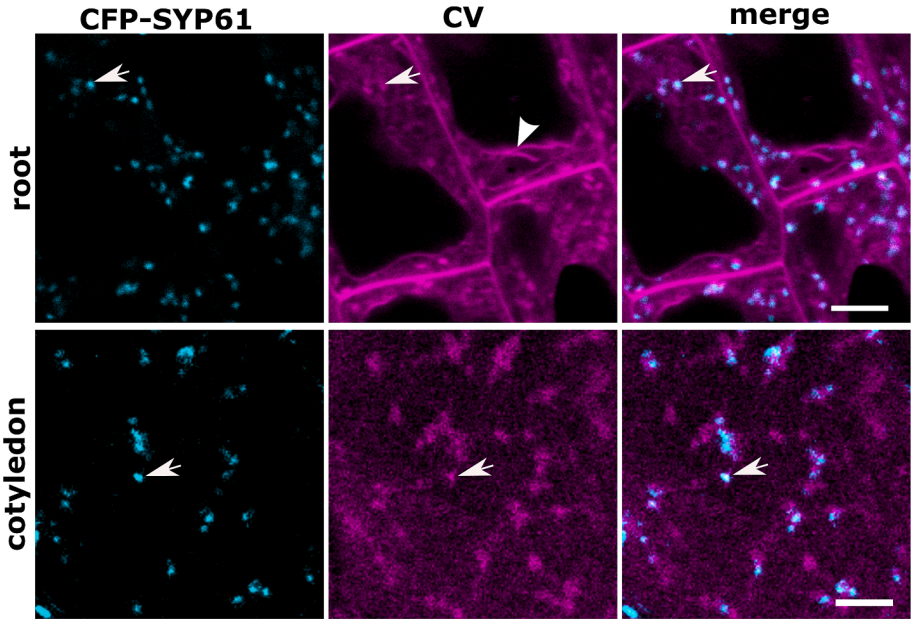


**Fig. 3.** Confocal images showing an association of CFP-SYP61 in root (top panel) and cotyledon epidermal cells (bottom panel) of *Arabidopsis thaliana* Col-0 and cresyl violet. White arrowhead and arrow, indicate vacuole and endosome respectively. Scale bar, 5 μm.

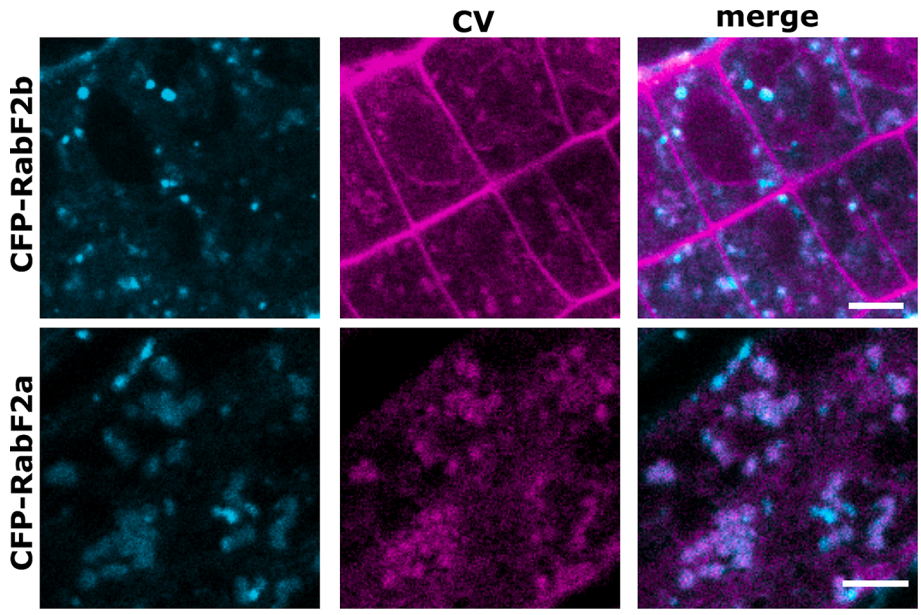


**Fig. 4.** Confocal images showing an association of CFP-RabF2a or CFP-RabF2b in cotyledon epidermal cells of *Arabidopsis thaliana* Col-0 and cresyl violet. Scale bar, 5 μm.

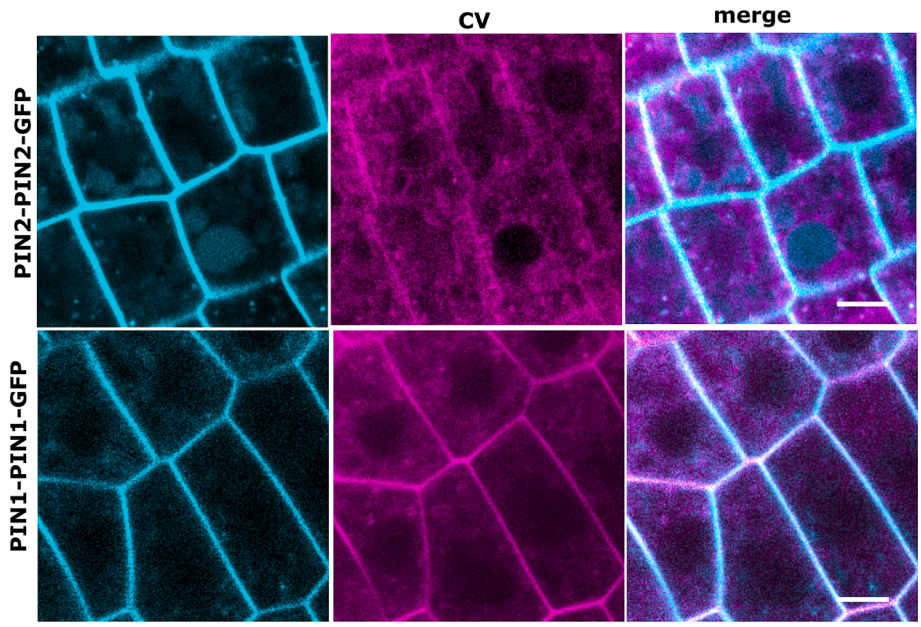


**Fig. 5.** Confocal images showing an association of PIN1-PIN1-GFP or PIN2-PIN2-GFP in root cells of *Arabidopsis thaliana* Col-0 and cresyl violet. Scale bar, 5 μm.

Network) TGN/EE which is characterized by higher acidic properties compared to the rest of the Golgi stacks. The optimal concentrations of cresyl violet that we found did not disrupt the integrity of the endomembrane compartments either affect cellular homeostasis. Furthermore, we did not observe any sign of toxicity or comparable adverse effects following the application of this fluorescent dyes on *A. thaliana* plants stably expressing ER-YK, ST-GFP, GFP-SYP61 and the Rabs proteins RabF2a and RabF2b. Indeed, no one of those cellular compartments appeared morphologically or dynamically compromised. An additional significant aspect that has been examined is the potential phototoxicity that may be induced by these chemical dyes, this is an important aspect especially during in vivo studies. Phototoxicity is a common issue with several other well-established fluorescent markers, which are prone to produce reactive oxygen species (ROS) when operating at shorter wavelengths. As a consequence, employing excitation wavelengths that are relatively high (543 nm) not only mitigate phototoxicity but also cause a reduced extent of the probe photobleaching.

## 3. Conclusions

The characterization of cresyl violet in plant cells revealed its usefulness for studying the organization of acidic compartments in vivo across different plant organs. It exhibits several distinct advantages under all tested conditions and with various plant samples. This dye it is particularly useful for studying cellular compartment like endosomes, which are challenging organelles because of their dynamic and diverse identity [3,19]. Cresyl violet resulted to fully label the all the endosomal populations, even better than the other two conventional endosomal marker FM4-64 and FM1-63 that only label certain endosome

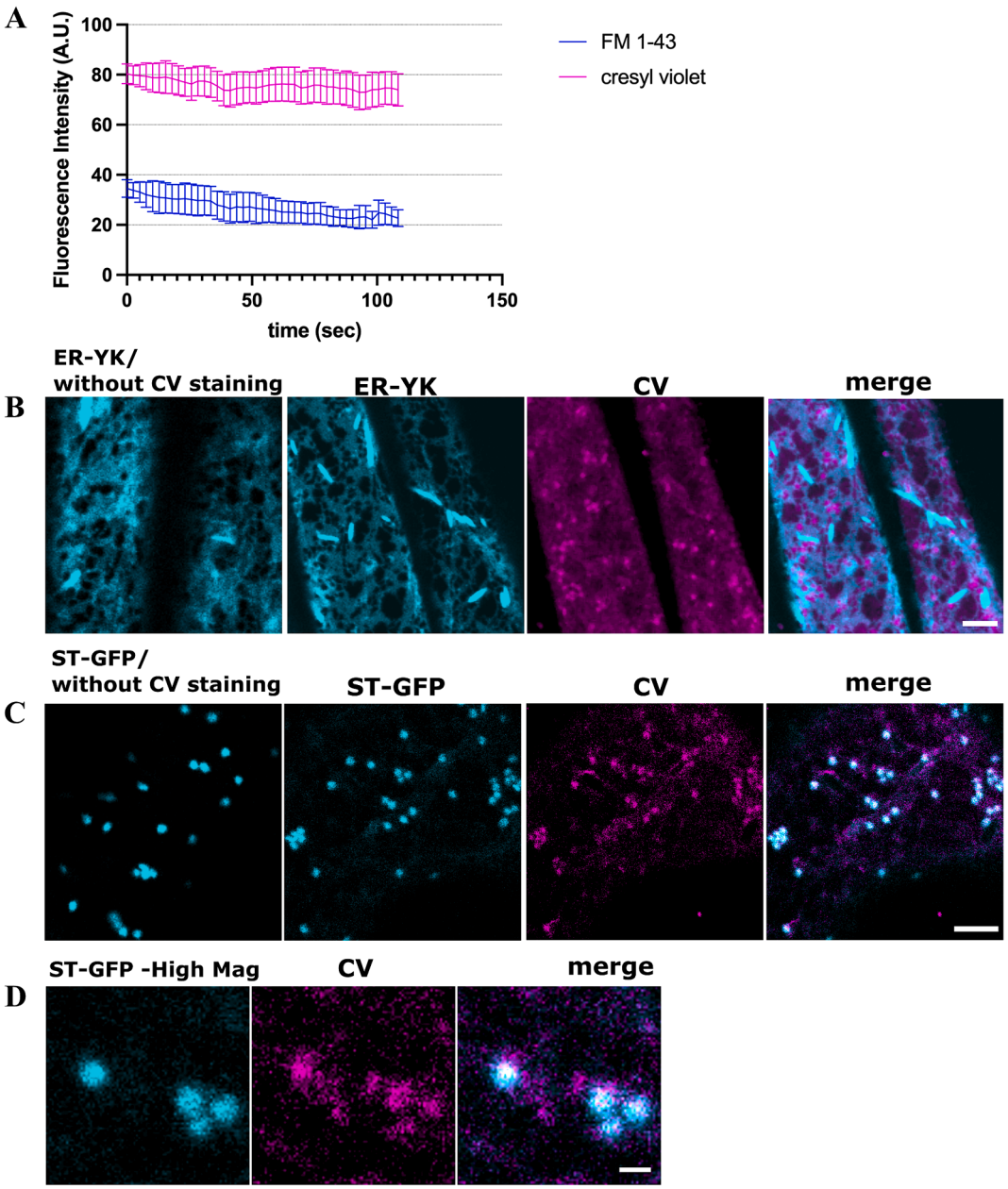


**Fig. 6.** Panel A, Photostability curve of cresyl violet compared to that of FM1-43. Panel B, C, D, Subcellular distribution of ERYK (A) and STGFP (B) in *Arabidopsis thaliana* Col-0 in presence of cresyl violet. (C) High magnification show distribution of cresyl violet on a portion of Golgi labeled by STGFP. Scale bar, 5 μm.

populations, and more efficiently for fast localization investigation compared to the reported molecular protein fusions available as molecular marker such as SYP61 and Rabs.

Furthermore, an overview and map of the different acidic compartments or regions can be gained, discriminating between the most and least acidic cellular bodies. Even within a single endosome or labeled compartment, it will be possible to visualize the most acidic portions. Labeling all endosome populations in plant cells and comprehensively understanding vesicle trafficking remain significant obstacles in this field. To this end, this marker will yield groundbreaking new insights.

Several are the advantage of using cresyl violet: cyto-safety, it is indeed not cytotoxic; photostability making it an ideal marker for timelapse acquisitions; pH resistant, its fluorescence varies on pH level making of it a good marker for acidic compartments and a good indicator for pHs ranges.

The last aspect is particularly interesting when the pH of plants needs to be followed for physiological studies. Indeed, plant cell metabolism, growth, and development can be influenced by fluctuations in pH, it is therefore essential to be able to monitor pH levels and changes occurring within specific compartments of various plant tissues, including mesophyll, guard cells, phloem, vascular bundles, root hairs, pollen tubes or at endomembrane/ organelle level. Quick changes in pH function as a control mechanism within the cell, regulating communication between organelles and leading to cell homeostasis and function.

Lastly, and by no means least, cresyl violet is an exceptionally inexpensive product, this dye can be suitable for extensive screening that would otherwise result less affordable for large sample size analysis. When taking into account the quantities necessary for each experiment and to obtain accurate measurements, cresyl violet costs 30,000 times less than the probes that are most frequently mentioned in the scientific literature [14].

Live-cell imaging in plant cell biology stands to gain significantly from this novel marker. Its exceptional performance across multiple applications allows for visualization of the complete organization of

acidic endomembrane compartments, including internalization and recycling processes within all acidic compartments.

## 4. Materials and methods

### 4.1. Plant material

*Arabidopsis thaliana* Col-0 and Col-0 plants expressing endomembrane fluorescent markers Syp61-GFP, RabF2a, RabF2b, VACYFP, STGFP, ERYK were used in this study for confocal microscopy analysis. Arabidopsis seedlings were grown with a cycle of 16 h light/8h dark regime at respectively 22 °C and 20 °C keeping humidity constant at 60 %.

### 4.2. Dye concentration and storage

Cresyl violet acetate was purchased from Sigma-Aldrich, the stock was prepared at concentration of 50 mM in DMSO, aliquoted in 1.5 ml tubes to reduce cycle of thawing.

FM1-43 was purchased from thermofisher. The stock was prepared at concentration of 500 μM in DMSO, then aliquoted.

### 4.3. Cresyl violet and FM1-43 treatment

Seedlings of Arabidopsis, which were six days old, were incubated for five minutes in 0.5 × LS media supplemented with 50 μM cresyl violet. Following this, the seedlings were washed three times at room temperature, mounted, and examined. Epidermal cells from the root tip and/or leaf of the cresyl violet-stained seedlings were subjected to confocal microscopy investigations. For FM1-43 labelling in Arabidopsis, a five-minute incubation period of six days old Arabidopsis seedlings in 0.5 × LS media supplemented with 2 μM FM1-43 was applied. After the incubation seedlings were mounted on microscope slide and epidermal cells from the root tip and leaf of -stained seedlings were subjected to confocal microscopy analysis.

### 4.4. Cresyl violet characterization in plant cells

Fluorescence spectra relative to cresyl violet and FM1-43 were collected using an upright microscope stand and a Leica TCS SP5 confocal microscope (Leica Microsystems CMS, Wetzlar, Germany) equipped with an acusto-optical beam splitter (DMI6000). A 40x objective (HCX PL APO OIL UV) was employed to capture an image of a 246x246 mm area, with a pixel spatial calibration of 0.5 mm. The value of the pinhole was one “airy unit.” The fluorescence spectra of cells stained with cresyl violet and FM1-43 were acquired using the Leica LAS-AF software package, spanning the 500–640 nm waveband. The measurements were performed in the λ-scan mode, with a detection window of 10 nm.

The fluorescence emission spectra of 50 μM cresyl violet were analyzed in the presence of LS medium at various pH solutions in order to identify variations in the behavior of the fluorescence intensity.

### 4.5. Fluorescence microscopy

Confocal images acquisition was performed using a Leica SP5 confocal microscope on root and cotyledon epidermal cells (Arabidopsis) equipped with a 40x objective (HCX PL APO OIL UV). Imaging of markers Syp61-GFP, CFP-RabF2a, CFPRabF2b, vacYFP, STGFP, ERYK was achieved as follow, for CFP and GFP blue shifted excitation a 458 laser line and fluorescence signal emission acquired between 475 and 495 was used, for YFP imaging it was used as laser line excitation 514 nm and emission was detected between 560 and 600. For cresyl violet it was used 543 nm as excitation laser line and 635–655 as emission detection window. A minimum of five separate experiments were conducted to obtain the results that are provided in this study.

### 4.6. Statistics

One-way ANOVA analysis was employed in conjunction with Tukey’s post tests to analyze the data. Asterisks denote significance in statistical analysis: Not significant; ***P,0.001; **0.001.P,0.01; *0.01.P,0.05; n.s. Error bars represent the s.e.m.; Inkskape was implemented to manipulate the images.

### CRediT authorship contribution statement

**Luciana Renna:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Mattia Cataudella:** Formal analysis, Investigation. **Elisa Masi:** Resources. **Alessio Papini:** Resources. **Stefano Mancuso:** Resources. **Giovanni Stefano:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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### Software

Software used for image processing was the Fiji/ImageJ suite.

### Availability of Biological materials

All fluorescent markers for plant organelles are available from ABRC stock center.

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