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ORIGINAL ARTICLE

Novel fluorochromes label tonoplast in living plant cells and reveal changes in vacuolar organization after treatment with protein phosphatase inhibitors

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Abstract

The recently synthesized isocyanonaphtalene derivatives ACAIN and CACAIN are fluorochromes excitable at wavelengths of around 366 nm and bind cysteine-rich proteins with hydrophobic motifs. We show that these compounds preferentially label tonoplasts in living *Arabidopsis* and tobacco (*Nicotiana tabacum* SR1) cells. ACAIN-labeled membranes co-localized with the GFP signal in plants expressing GFP-δ-TIP (TIP2;1) (a tonoplast aquaporin) fusion protein. ACAIN preserved the dynamics of vacuolar structures. *tip2;1* and triple *tip1;1-tip1;2-tip2;1* knockout mutants showed weaker ACAIN signal in tonoplasts. The fluorochrome is also suitable for the labeling and detection of specific (cysteine-rich, hydrophobic) proteins from crude cell protein extracts following SDS-PAGE and *TIP* mutants show altered labeling patterns; however, it appears that ACAIN labels a large variety of tonoplast proteins. ACAIN/CACAIN could be used for the detection of altered vacuolar organization induced by the heptapeptide natural toxin microcystin-LR (MCY-LR), a potent inhibitor of both type 1 and 2A protein phosphatases and a ROS inducer. As revealed both in plants with GFP-TIP2;1 fusions and in wild-type (Columbia) plants labeled with ACAIN/CACAIN, MCY-LR induces the formation of small vesicles, concomitantly with the absence of the large vegetative vacuoles characteristic for differentiated cells. TEM studies of MCY-LR-treated *Arabidopsis* cells proved the presence of multimembrane vesicles, with characteristics of lytic vacuoles or autophagosomes. Moreover, MCY-LR is a stronger inducer of small vesicle formation than okadaic acid (which inhibits preferentially PP2A) and tautomycin (which inhibits preferentially PP1). ACAIN and CACAIN emerge as useful novel tools to study plant vacuole biogenesis and programmed cell death.

Keywords ACAIN/CACAIN · *Arabidopsis* · Tonoplast · Hypocotyl · Microcystin-LR · Vacuolar organization

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Introduction

Recently, two novel fluorochromes, 1-(2-acryloyloxy-3-chloro-prop-1-yl)-amino-5-isocyanonaphthalene (ACAIN) and its chlorinated derivative (2-chloroacryloyloxy-3-chloroprop-1-yl)-amino-5-isocyanonaphthalene (CACAIN) (Fig. 1), were synthesized in our laboratory. These are low MW molecules (315 Da for ACAIN) (Nagy et al. 2016). They have characteristic absorption spectra in the UV wavelength range with a peak at 366 nm for ACAIN, but absorption persists up to 405 nm. They are excitable with emissions at the blue and green wavelength range when a stock dissolved in DMSO is diluted in water (peaks at 423 and 502 nm) (Nagy et al. 2016). Moreover, ACAIN and its more reactive derivative CACAIN proved to bind lysil-alanyl-cysteine (KAC) as well as bovine serum albumin at hydrophobic motifs (Nagy et al. 2016). Due to the hydrophobic nature of these compounds, we suspected they have the potential to penetrate easily through biological membranes and label intracellular structures containing proteins with hydrophobic motifs.

Microcystin-LR (MCY-LR) is a natural cyclic heptapeptide toxin produced by several cyanobacterial genera. It is known to be a potent inhibitor of the serine-threonine protein phosphatases, mainly of type 1 and 2A (PP1 and PP2A), and of PP3, PP4, PP5, and PP6 (Swingle et al. 2007). It is also a strong inducer of reactive oxygen species (ROS) (Bouaïcha and Maatouk 2004; Campos and Vasconcelos 2010). Due to these two properties, it affects key cellular events: metabolic regulation, signal transduction events, and cell-cycle regulation. Thus, it affects eukaryotic subcellular structures: chromatin, cytoskeletal, and endomembrane (mainly ER) organization (Chen and Xie 2016; Máthé et al. 2013, 2016). For this reason, it is ideal for the study of alterations of subcellular organization under stress. If ACAIN and CACAIN can label specific subcellular structures, MCY-LR could be ideal to be used as a tool for the detection of changes highlighted by these fluorochromes.

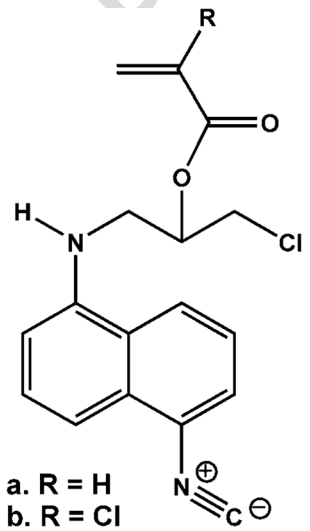

Fig. 1 Chemical formulae of ACAIN (a, R = H) and CACAIN (b, R = Cl)

In the light of the above arguments, our main goals were (1) to detect the subcellular structures labeled by ACAIN and CACAIN and to assess whether they can label proteins related to those subcellular structures, and (2) to test these fluorochromes for their suitability to detect stress-induced subcellular alterations induced by MCY-LR. We show that ACAIN/CACAIN are suitable as alternative labels for specific cellular structures—tonoplasts—and for detecting changes in the organization of tonoplast-coated endomembranes.

Materials and methods

Chemicals

ACAIN/CACAIN were synthesized as we have previously described (Nagy et al. 2016). Briefly, the synthesis started from 1,5-diaminonaphthalene (Sigma-Aldrich, St. Louis, MO, USA) by Hofmann isonitrile synthesis using chloroform and potassium hydroxide, followed by the addition of epichlorohydrin on the amine group. Secondary hydroxyl group formed was then acylated with acryloyl chloride (ACAIN) or 2-choloroacrylic acid (CACAIN). MCY-LR was purified from *Microcystis aeruginosa* BGSD243 according to the procedure of Kós et al. (1995) modified by Vasas et al. (2004). Briefly, methanolic extracts of *M. aeruginosa* cells were vacuum evaporated, re-extracted with 10 mM Tris–HCl, pH 7.5, and subjected to ion-exchange chromatography on DEAE-cellulose (DE-52; Whatman, Maidstone, UK) columns. This was followed by desalting and semi-preparative HPLC to obtain MCY-LR of purity ≥ 95%.

Plant material and treatments

Arabidopsis thaliana genotypes used were Col-0 (wild-type), plants bearing GFP-TIP2;1 (δ-TIP) fusion protein described by Cutler et al. (2000)), and four knockout mutants in Col-0 background of tonoplast intrinsic proteins (TIPs). These were *tip1;1*, *tip1;2*, *tip2;1*, and their triple mutant obtained as described previously (Reinhardt et al. 2016). The tobacco genotype used was *Nicotiana tabacum* SR1. Seeds were surface sterilized by rinsing two times for 10 min with 10% of a sodium-hypochlorite-containing solution, then washed four to five times for 5 min with sterile water. Seeds were transferred to Murashige–Skoog (MS) basal medium supplemented with Gamborg's vitamins, 2% (*w*/*v*) sucrose (Molar, Budapest, Hungary), and 0.8% (*w*/*v*) Bacto-agar (Difco, Lawrence, KS, USA) (Gamborg et al. 1968; Murashige and Skoog 1962). After a 48-h cold treatment (used for *Arabidopsis*), plates were placed in a tissue culture room

(14:10 h photoperiod, 22 ± 2 °C, 60 μmol m^{-2} s^{-1} photon flux density in the light period). After 1 week of culture, part of the seedlings was directly subjected to ACAIN/CACAIN cytological labeling or ACAIN staining of root and shoot extracts after SDS-PAGE (see sections below). For detecting ACAIN-labeled proteins on SDS-PAGE, *Lemna minor* shoots axenically subcultured for 1 week on liquid Allen medium (Allen 1968) were used as well. Part of *Arabidopsis* (Col-0 and GFP-TIP2;1) seedlings were further treated with protein phosphatase inhibitors. Col-0 and GFP-TIP2;1 seedlings were treated with 1–5 μM MCY-LR for 4–72 h. Col-0 plants were further stained with ACAIN/CACAIN. GFP-TIP2;1 seedlings were also treated with 1 μM okadaic acid (OA) for 24 h and 1 μM tautomycin (Tm) for 4–24 h.

ACAIN/CACAIN staining and microscopy

Control and protein phosphatase-treated whole seedlings were placed in four-well tissue culture chambers for microscopy (Sarstedt, Nümbrecht, Germany) and washed with phosphate buffered saline (PBS). ACAIN/CACAIN stocks dissolved in DMSO were diluted in PBS with or without 0.05% (*v*/*v*) Triton X-100 (Reanal, Budapest, Hungary) to obtain final concentrations of 20 μg mL^{-1}, used for labeling of seedlings. Duration of labeling was 30 min, followed by three consecutive 5-min washes with PBS. Samples were then first visualized with a conventional Olympus Provis AX-70 (Olympus, Tokyo, Japan) fluorescence microscope at excitation wavelength of 320–360 nm. This was followed by CLSM analysis. ACAIN/CACAIN stained Col-0 and GFP-TIP2;1 seedlings were analyzed with a Zeiss LSM 880 confocal microscope by using a × 40 C-Apochromat water immersion objective. Excitation and emission wavelengths were 405/408–473 nm (ACAIN/CACAIN), 488/492–540 nm (GFP), and 543/547–614 nm (chlorophyll autofluorescence). For treatment of GFP::TIP2;1 seedlings with protein phosphatase inhibitors (without ACAIN/CACAIN labeling), a Leica TCS-SP5 confocal microscope/HCX APO L U-V-I 40.0 × 0.80 water immersion objective was used. GFP was excited with a 488 nm Ar laser, and emission was at 503–515 nm. For chlorophyll autofluorescence detection, the 543 HeNe laser was used. Emission was at 650–710 nm, and chlorophyll autofluorescence was pseudocolored in blue on the GFP-chlorophyll composite images.

All cytological experiments were performed at least three times, and representative results are presented ("Results" section).

TEM studies

Col-0 seedlings were treated with 1–2 μM MCY-LR for 72 h (see above). TEM preparations were made as follows: segments of control and microcystin treated *Arabidopsis thaliana* hypocotyls were fixed in 2% glutaraldehyde for 3 h and in 1% osmium tetroxide for 2 h (both fixatives were dissolved in 0.1 M K–Na–phosphate buffer, pH 7.2) rinsed in the buffer and embedded in Durcupan resin after dehydration in an ethanol series ended with propylene oxide. Ultra-thin sections (70 nm) were cut with a Reichert Ultracut E ultramicrotome (Leica Microsystems, Vienna, Austria), mounted on uncoated Cu/Pd grids (Polysciences, Warrington, PA, USA), and stained with 2% uranyl acetate dissolved in methanol for 4 min and lead citrate for 6 min. Sections were studied with a Hitachi 7100 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 kV.

ACAIN labeling of proteins following SDS-PAGE

Protein extracts from shoots and roots of *Arabidopsis* and tobacco seedlings as well as *Lemna minor* shoots were prepared in the SDS (Sigma-Aldrich) containing standard Laemmli buffer (Laemmli 1970), but without boiling to allow further renaturation of proteins (see 2-propanol treatments below). To check whether this extraction does not allow protein degradation, alternatively extracts were prepared in the presence of a protease inhibitor cocktail (0.5% *v*/*v*; Roche Applied Science, Indianapolis, USA) as well as without protease inhibitors and with standard protein boiling. Protein contents of samples were assayed by the Bradford (1976) method. Extracts containing 40 μg protein per well were loaded along with molecular weight markers (Sigma-Aldrich, BLUeye Prestained Protein Ladder). SDS-containing gels were prepared essentially by the Laemmli (1970) method except that 7.5–18% polyacrylamide gradient (running) gels proved to be the best for our purposes and used accordingly. After SDS-PAGE, gels were rinsed for 10 min in sterile water, then twice for 10 min in 20% (*v*/*v*) 2-propanol (VWR, Radnor, USA) and twice for 20 min in PBS. Afterwards, labeling was performed for 5 h with 35 μg mL^{-1} ACAIN in PBS. Gels were then washed for 10 min with 20% 2-propanol, followed by two consecutive washes with PBS for 5 min each. ACAIN-labeled proteins were detected by illumination at 365 nm and photographed with an UVITEC transilluminator/gel documentation equipment (UVITEC, Cambridge, UK). MW of proteins was determined with the UVITEC® software. Band intensities and intensity curves were determined with the aid of the GelAnalyzer 2010 software. Besides ACAIN staining, gel slices containing the same protein samples were stained with 0.2% Coomassie Brilliant Blue (Serva, Heidelberg, Germany) for 4 h.

All SDS-PAGE work was performed in three independent experiments and images of representative gels are shown in the "Results" section.

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Results

ACAIN labels tonoplasts in different plant species and TIPs contribute to this labeling pattern

When living Col-0 plants were stained with ACAIN and visualized with a conventional fluorescence microscope, specialized shoot epidermal cells (trichomes and guard cells) as well as hypocotyl cortex cells showed endomembrane labeling. Labeling pattern raised the possibility of ACAIN binding to vacuolar membranes (Fig. 2a–d). Therefore, in the next step, we labeled whole *Arabidopsis* seedlings expressing GFP-TIP2;1, known for the characteristic tonoplast GFP signal (Cutler et al. 2000). ACAIN and GFP signals showed a perfect co-localization in hypocotyls (Fig. 2e). Labeling of Col-0 and GFP-TIP2;1 seedlings showed the presence of large vacuoles as well as smaller tonoplast-coated vesicles in hypocotyl epidermal and cortex cells (Fig. 2e, f).

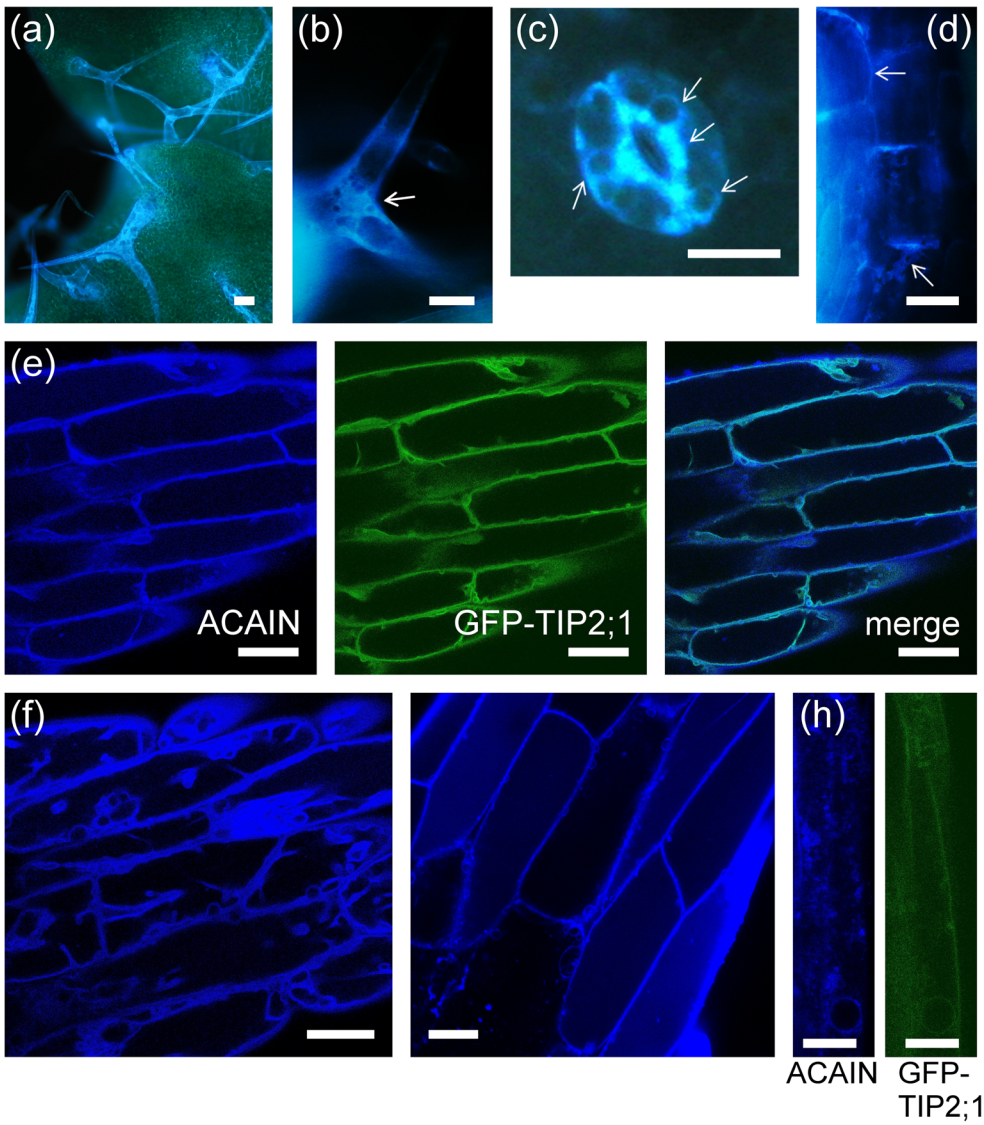

Fig. 2 ACAIN labels exclusively tonoplasts in living plant cells. All images were taken from *Arabidopsis* seedlings, except (**g**) that was of hypocotyls from *N. tabacum* SR1 seedlings. Images of (**a**)–(**d**) are of conventional fluorescence microscopy with arrows indicating the labeling of membrane structures. (**e**)–(**h**) are CLSM images. ACAIN labeling (**a**) of trichomes, (**b**) detail of trichome labeling, (**c**) of hypocotyl guard cells, and (**d**) of hypocotyl cortex cells from Col-0 plants. Conventional fluorescence microscope images show the probability of endomembrane labeling. **e** ACAIN labeling of a Triton X-100 permeabilized hypocotyl segment of *Arabidopsis* Col plants containing GFP-tagged δ-TIP (TIP2;1) protein. ACAIN label co-localizes with TIP2;1. **f**, **g** ACAIN labeling of hypocotyl cells not permeabilized with Triton X-100 from *Arabidopsis* Col-0 (**f**) and *N. tabacum* SR1 (**g**). **h** ACAIN labeling of Triton X-100 permeabilized differentiated cells of root from *Arabidopsis* plants containing GFP-tagged δ-TIP (TIP2;1) protein. Both ACAIN and GFP signals are diffuse, showing multiple tonoplast-coated vesicles. Scale bars: 30 μm

To exclude the possibility that ACAIN labels only tonoplasts because it cannot penetrate into other subcellular structures, ACAIN labeling was performed in the presence of increased DMSO concentration (10%) and/or 0.05% Triton X-100 as well, but these well-known compounds that increase membrane permeability did not change the labeling pattern (compare Fig. 2e and f). ACAIN could label tonoplasts in hypocotyl cells of tobacco (*N. tabacum* SR1) as well, in a similar manner to *Arabidopsis* (Fig. 2f, g). The fluorochrome preserved cell viability and vacuolar dynamics, thus it proved to be suitable for live cell imaging (Supplementary Movie 1). For differentiated or meristematic cells of *Arabidopsis* roots, labeling was weaker and more diffuse, but this was true for the GFP signal in GFP-TIP2;1 roots as well (Fig. 2h). Concerning specificity of ACAIN for tonoplast, other endomembrane structures like the ER or Golgi apparatus had fluorescent signals clearly different to ACAIN labeling in transgenic *Arabidopsis* plants with GFP- or YFP-fusion proteins specific for those organelles (Supplementary Fig. 1a–c). Plasmolyzed hypocotyl cells of YFP-PIP2a plants (with YFP signal of the plasma membrane) showed nearly exclusive YFP label of Hechtian strands and exclusive ACAIN label of shrunk vacuoles (Supplementary Fig. 1d, e). Plants with ER/Golgi/plasma membrane YFP/GFP signals were from Boevink et al. (1998), Cutler et al. (2000), Mathur et al. (2003), and Nelson et al. (2007)).

Different TIP knockout mutants showed altered ACAIN labeling in hypocotyls as compared to Col-0 plants. *tip1;1* and *tip1;2* mutants showed weaker, but still well-detectable tonoplast labeling (compare Fig. 3a–c). For *tip2;1* and the triple *tip1;1-tip1;2-tip2;1* mutants, ACAIN signal was not detectable at excitation settings identical to Col-0 seedlings, but brightness–contrast adjustment showed that it still labeled tonoplasts, although the signal was very weak (Fig. 3d, d′, e, e′).

ACAIN labels proteins on SDS-polyacrylamide gels

The extraction of proteins in Laemmli buffer—due to its SDS content (see "Materials and methods" section)—allowed obtaining of crude cellular protein extracts that contained membrane proteins. For Col-0 seedlings, shoot extracts contained several proteins that were labeled by ACAIN: the most characteristic bands were proteins of 27 (band 1), 24 (band 2), 19, and 18 kDa (double band 3a and 3b) (see arrows on Fig. 4a). Small (below 11 kDa) and larger (≥ 35 kDa) MW proteins could also be detected (Fig. 4a). We could not detect ACAIN-labeled proteins in Col-0 root extracts (Fig. 4a). Concerning shoot extracts of *TIP* mutants, the general labeling pattern was weaker in *tip1;1* and *tip2;1* as compared to controls (Fig. 4a). Shoot extracts of *N. tabacum* SR1 and *L. minor* contained ACAIN-labeled proteins as well. The most characteristic band had a MW of 24 kDa for both extracts (Fig. 4b, arrow) and several other proteins showed weak labeling (Fig. 4b). To check whether the protein extraction method without boiling (see "Materials and methods" section) does protect

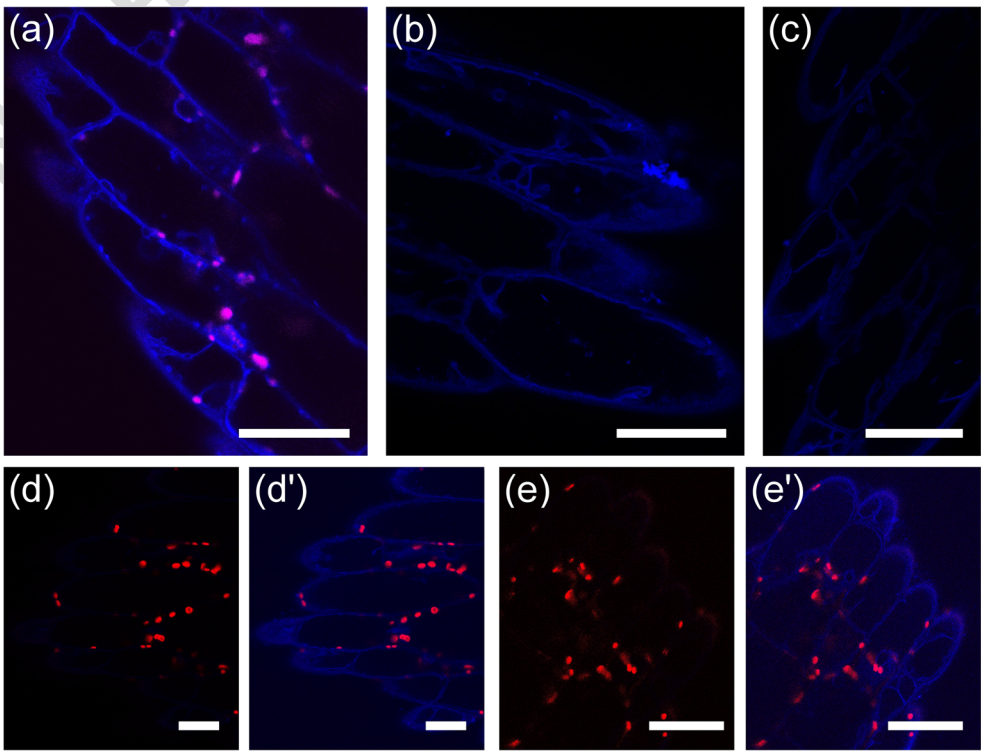

Fig. 3 ACAIN labeling in the absence of Triton X-100 in hypocotyls of *TIP* knockout mutants reveals weaker, but still existent tonoplast signal. Images of (**a**–**d**, **e**) were taken with the same gain and laser intensity settings. **a** Col-0, ACAIN labeling, and chloroplast autofluorescence; **b** *tip1;1*; **c** *tip1;2*; **d** *tip2;1*, ACAIN labeling and chloroplast autofluorescence. (**d′**) is the brightness/contrast adjusted version of (**d**) to show that the ACAIN signal is still present. **e** ACAIN labeling and chloroplast autofluorescence in the triple TIP knockout mutant. (**e′**) is the brightness/contrast adjusted version of (e) to show that the ACAIN signal is still present. Scale bars: 30 μm

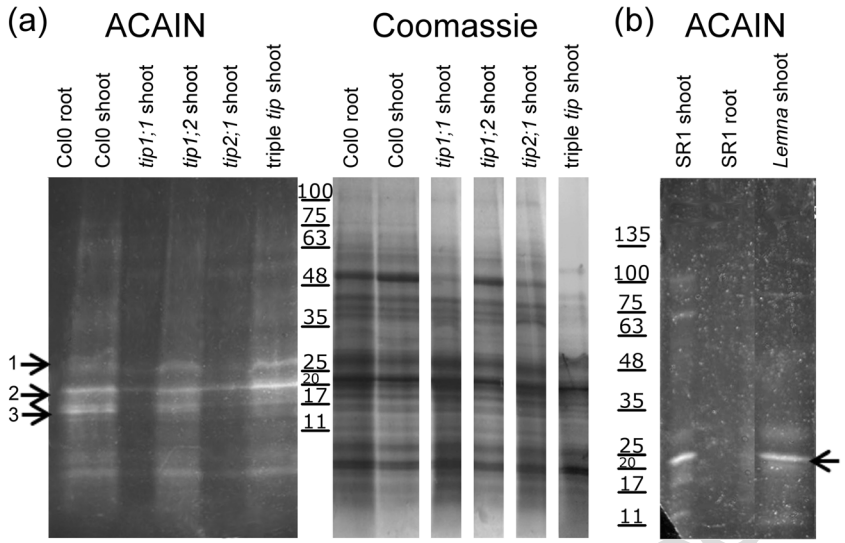

Fig. 4 ACAIN labels seedling proteins as revealed by SDS-PAGE. **a** ACAIN label of *Arabidopsis* (Col-0) roots and shoots (no labeling of roots), of shoots from *Arabidopsis* TIP mutants, and the corresponding total protein patterns (Coomasie Blue staining) along with a molecular weight marker. Arrows indicate the most important changes in the intensity of protein bands from mutants as compared to the wild-type control. Arrow 1, 27-kDa protein; arrow 2, 24-kDa protein; arrow 3, double band of 18–19-kDa proteins. **b** ACAIN labels proteins of shoots from young tobacco (*N. tabacum* SR1) seedlings and *L. minor* shoots, but not roots of tobacco seedlings. Arrow indicates the most characteristic protein band of 24 kDa

extracts from being degraded by proteases, we analyzed Col-0 extracts prepared in the presence of a protease inhibitor cocktail as well as boiled extracts. We could see no difference in protein patterns as compared to the samples prepared without boiling and protease inhibitors (Supplementary Fig. 2). This statement is valid for tobacco and duckweed extracts as well (data not shown).

Detailed analysis of intensity curves of SDS gels with *Arabidopsis* ACAIN-labeled protein bands showed the following changes in protein band patterns (Fig. 5): (1) for the 27- and 24-kDa proteins (bands 1–2), intensity in *tip1;1* and *tip2;1* shoots decreased as compared to Col-0 shoots, while they remained unchanged in *tip1;2* shoots and increased in the triple TIP mutant (see arrows 1–2 on Fig. 4a). (2) for the 18–19-kDa proteins (double band 3a and 3b), the presence of two proteins with similar MWs was clearly visible in Col-0 shoots (see inset box for Col-0 on Fig. 5). This double band nearly disappeared in *tip1;1* extracts and its intensity decreased in general in mutants. Moreover, one band was apparently missing in *tip2;1* and triple TIP mutants (arrow 3 on Fig. 4a and inset box for triple mutant on Fig. 5).

ACAIN/CACAIN labeling reveals changes in vacuolar organization induced by protein phosphatase inhibitors

ACAIN and CACAIN staining gave identical labeling patterns of tonoplasts in Col-0 hypocotyls (Fig. 6a, b). CACAIN labeling of MCY-LR treated hypocotyls for relatively short term (24 h) showed the absence of large vacuoles in many cortex cells. Instead, small, tonoplast-coated vesicles were present (Fig. 6c). Many of these vesicles engulfed chloroplasts (Fig. 6c, arrows). However, engulfment of plastids by smaller tonoplast-coated vesicles could be observed in some control cells as well (data not shown). ACAIN labeling of MCY-LR-treated cells showed similar changes (data not shown).

GFP signal in hypocotyls from GFP-TIP2;1 seedlings treated with protein phosphatase inhibitors showed the following: controls showed normal vacuolar system (Fig. 6d). Four hours of MCY-LR treatment revealed the absence of large vacuoles and the presence of small tonoplast-coated vesicles similarly to the pattern revealed by CACAIN/ACAIN staining (Fig. 6e). Moreover, the movement/dynamics of these vesicles was very slow (Supplementary Movie 2). OA treatments did not alter vacuolar organization (Fig. 6f). In contrast, in the presence of Tm, the proportion of large vacuoles decreased in many cells (while for MCY-LR, large vacuoles were completely lacking in several cells), and this effect increased over time (9 vs. 24 h) (Fig. 6g–i). Thus, Tm did not have such dramatic effects on vacuolar organization as MCY-LR.

TEM studies showed normal organization of endomembranes in control Col-0 plants, with large vacuoles and smaller, abundant vesicles (Fig. 6j). After 72 h of treatment with 1–2 μM MCY-LR, an increase in the abundance of multimembrane vesicles as well as autophagosome-like structures incorporated into the large vacuoles were observed (Fig. 6k; Supplementary Fig. 3). Apparently, multimembrane vesicles formed in the cytosol (Fig. 6k) were later incorporated in the large vacuoles (Supplementary Fig. 3a).

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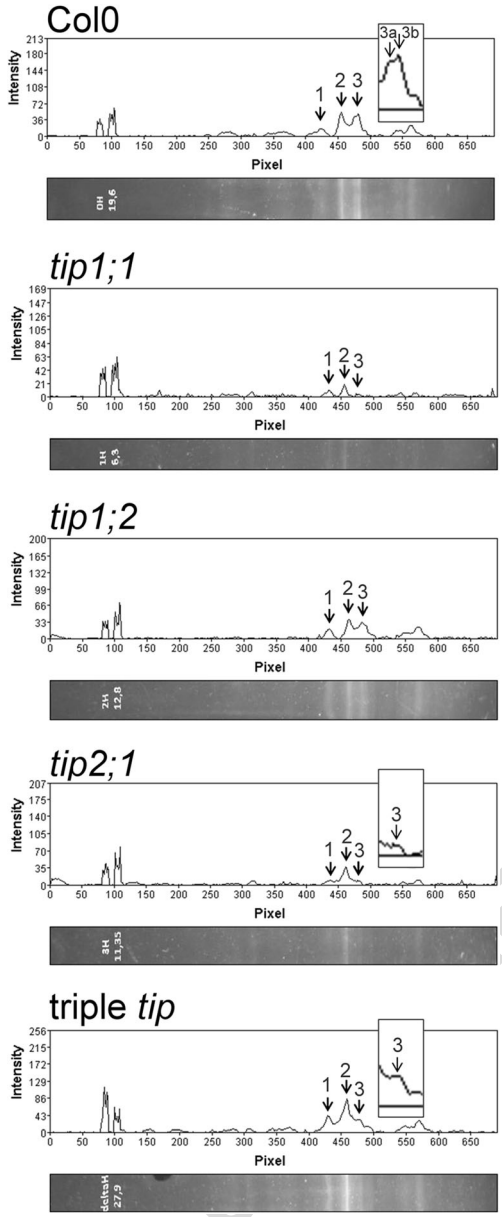

Fig. 5 Intensity curves of *Arabidopsis* wild-type and TIP mutant shoot protein patterns upon ACAIN labeling; analysis of the gel presented in Fig. 4a. Arrows show the intensity of characteristic bands indicated on Fig. 4a. Band of the 27-kDa protein (arrow 1) shows relatively low intensity except the triple TIP mutant, where its labeling intensity increases. Band of the 24-kDa protein (arrow 2) shows decreased intensity for *tip1;1*, *tip1;2* and *tip2;1* relative to Col-0 and increased intensity in the triple TIP mutant. The double band of 18–19-kDa proteins (arrow 3) shows relatively high intensity for the Col-0 shoots (see inset box for the presence of two bands). All mutants show decreased intensity of labeling of this band, with its near absence in *tip1;1*. For *tip2;1* and the double TIP mutant, apparently there is only one band (see inset boxes)

Q2

Discussion

ACAIN and CACAIN label preferentially tonoplasts in living hypocotyl cells of *Arabidopsis* and tobacco, in a similar manner (Figs. 2f, g and 6a, b). Tonoplast labeling is proven by the co-localization of fluorochrome signals with GFP signals from *Arabidopsis* plants expressing GFP-TIP2;1 fusion protein (Fig. 2e) reported to highlight all endomembranes delimited by a tonoplast (Cutler et al. 2000). The second evidence for preferential tonoplast labeling is that Hechtian strands known to consist mainly of plasma membrane fractions that tend to attach to the cell wall are showing almost exclusively the YFP label when plasmolyzed hypocotyl cells of YFP-PIP2a plants (Cutler et al. 2000) are labeled with ACAIN (Supplementary Fig. 1d, e). Meanwhile, shrunk vacuoles of plasmolyzed cells probably delimited only by tonoplasts are labeled exclusively by ACAIN (Supplementary Fig. 1d, e). The surface of shrunken protoplasm is apparently showing both the ACAIN and YFP label (Supplementary Fig. 1e). However, one should note that during plasmolysis, tonoplast or segments of tonoplast may be tightly adhered to the plasma membrane (Oparka 1994). While the resolution of light microscopy/CLSM might not allow us to distinguish plasma membrane and tonoplast under these conditions, the possible tight adherence of the two membrane types may lead one to false co-localization. The third evidence is the different fluorescence signal pattern (as compared to ACAIN/CACAIN) for other endomembranes (ER, Golgi) in *Arabidopsis* plants with GFP/YFP-fusion proteins labeling those cell compartments (Supplementary Fig. 1a–c). The fourth evidence for the peculiar ACAIN/CACAIN labeling pattern is that the use of known surfactants (Triton X-100) neither increased the intensity of labeling nor caused labeling of other subcellular structures than tonoplasts (Fig. 2e–g). Data presented in the "Results" section suggest that the isocyanonaphthalene derivatives used in this study can be of broad use for tonoplast labeling in living plant cells. Since it works well in another model plant too (*N. tabacum* SR1), ACAIN/CACAIN labeling could be a good alternative to plants expressing GFP-fusion proteins for cytological studies on the tonoplast. It can be used for species where transgenic lines with fluorescent protein labels of tonoplasts are not available.

What could be the nature of proteins labeled by our isocyanonaphthalene derivatives? Theoretically, a large variety of proteins containing hydrophobic motifs must be labeled by ACAIN and its derivatives (Nagy et al. 2016). Among plant vacuolar membrane proteins, tonoplast intrinsic proteins (TIPs)—since they are aquaporins—are fitting, although not exclusively into this category. Indeed, two *Arabidopsis* knockout mutants (*tip2;1* and *tip1;1-tip1;2-tip2;1* triple mutant) showed much weaker signal than the wild-type (Col-0) plants as revealed by CLSM (Fig. 2). Thus, at least some of the TIPs are labeled by ACAIN (and CACAIN), but not exclusively TIPs are labeled. Tonoplasts are characterized by a large variety of channel proteins characterized by hydrophobic transmembrane domains (Batistič 2012; Maeshima 2001). Indeed, ACAIN staining of protein gels revealed a large number of

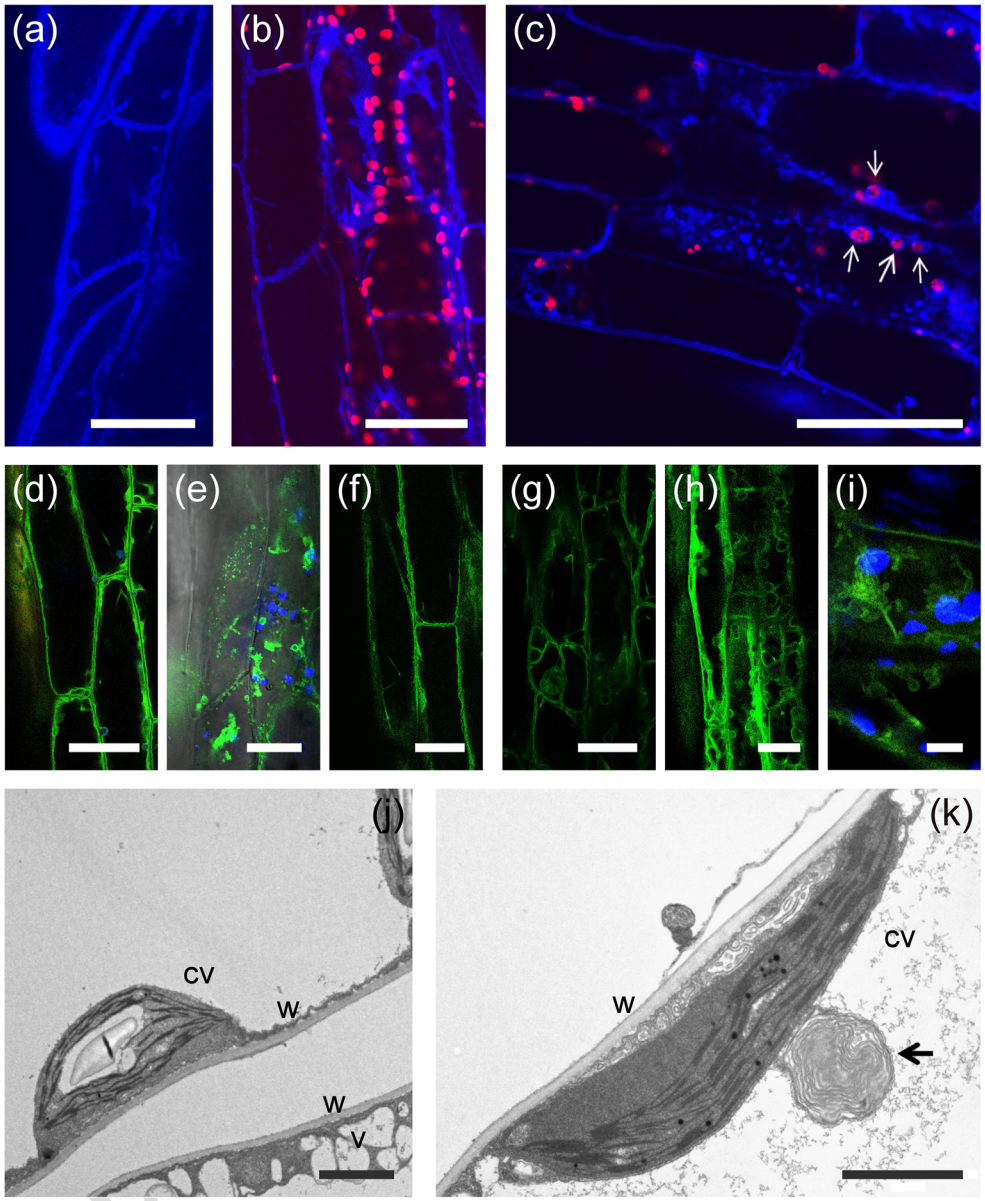

Fig. 6 CACAIN labeling in Col-0 (**a**–**c**) and analysis of GFP-TIP2;1 (**d**–**i**) hypocotyls reveal changes of vacuolar organization in hypocotyl cells treated with protein phosphatase inhibitors. **a** Control, ACAIN labeling, normal vacuolar system; **b** control, CACAIN labeling, tonoplasts (blue) and chloroplast autofluorescence (red); **c** treatment with 5 μM microcystin-LR (MCY-LR) for 24 h, CACAIN labeling. Disorganization of the vacuolar system and capturing of chloroplasts by tonoplast-coated vesicles (arrows). **d** Control, normal vacuolar system; **e** 1 μM MCY-LR, 4 h treatment showing numerous tonoplast-coated vesicles; **f** 1 μM okadaic acid (OA), 24 h—no visible changes in the vacuolar system. **g** Control in the presence of MS medium with 0.1% DMSO (tautomycin, Tm, treatments were made with stocks of the inhibitor diluted in DMSO). Treatments with 1 μM Tm for 9 h (**h**) and 24 h (**i**) show disorganization of the vacuolar system. Autofluorescence of chloroplasts is in blue for Fig. 5e and i. **j** TEM image of a control hypocotyl cell showing chloroplasts and normal endomembrane system; **k** TEM image of a hypocotyl cell treated with 2 μM MCY-LR for 3 days, showing a multimembrane vesicle (arrow). Legends for (**j**), (**k**): cv, central vacuole; v, vesicle belonging to the vacuolar system; w, cell wall. Scale bars: 30 μm (**a**–**h**), 10 μm (**i**), 2 μm (**j**, **k**)

labeled proteins. Among them, the 22–26-kDa TIPs (see Maeshima 2001 and Schüssler et al. 2008 for molecular weights) were presumably labeled, but several other proteins were also well detectable (Fig. 4). Interestingly, the intensity of ACAIN labeling decreased for the 22–26-kDa proteins in the *tip1;1* and *tip2;1* mutants, but increased in the triple knockout mutant (Figs. 3 and 4), predicting that for the latter mutant, the absence of three TIP proteins is compensated by increased expression of other proteins. It should be noted, however, that in roots, in general the absence of specific TIPs in the mutants is not compensated by other TIP isoforms (Reinhardt et al. 2016). Thus, our prediction might be valid only for shoot extracts, where ACAIN labeling of proteins following SDS-PAGE was clearly visible (Fig. 4) or for other

proteins than TIPs. It should be noted that to date, 10 proteins of the TIP family were detected in *Arabidopsis* (the expression of several TIPs is organ and tissue specific) and many other proteins with hydrophobic motifs are present in the tonoplast (Beebo et al. 2009). One of the most characteristic changes revealed by SDS-PAGE of the proteins from wild-type plants and mutants were the 18–19-kDa proteins (according to their MW, probably not TIPs) that were almost completely absent in the triple mutant (Figs. 4 and 5, "Results" section). At this stage, we can assume that our fluorochromes are suitable for the detection of specific proteins, among them tonoplast proteins in a crude cellular extract.

What is the uniqueness of ACAIN in the labeling of tonoplasts in live cells? There are several, relatively low molecular weight fluorochromes used for the labeling of plant vacuolar membranes. Different probes including pH markers, Lysosensor yellow/blue, glutathione/sulfhydryls, etc. were used for the labeling of vacuoles (mainly lytic vacuoles) in barley aleurone protoplasts (Swanson et al. 1998). Dyes of the FM family like FM4-64 can label tonoplasts, but other membrane types including those in animal cells (Golgi membranes, vesicles in the phragmoplast for plant cells during cytokinesis, or even the plasma membrane) are also labeled, thus their specificity is lower (Bolte et al. 2004). For plant cells, labeling of other membranes than tonoplasts by FM4-64 occurs after shorter labeling time (Bolte et al. 2004). In contrast, shorter ACAIN labeling times than 30 min (as specified in the "Materials and methods" section) will label tonoplasts preferentially, although labeling intensity is weaker (data not shown). This is probably due to the stable/covalent binding of the fluorochrome to certain tonoplast proteins, as it is known to establish stable bonds with hydrophobic proteins (Nagy et al. 2016) in contrast to FM4-64 known to be a partitioning dye that is preferentially binding to hydrophobic supramolecular structures like biological membranes. FM1–43, excitable at 488 nm, was described to be targeted to tonoplast over time and proven to be a good endocytosis marker in tobacco BY-2 cells (Emans et al. 2002). This dye is first targeted to the plasma membrane (Emans et al. 2002; Zhao and Dixon 2009), meaning that a short-term incubation does not label the tonoplast. Moreover, FM1–43 was proven to be cytotoxic for *Vicia faba* cells where it was used for plasma membrane and tonoplast labeling (Meckel et al. 2004). This is in contrast to our finding: ACAIN preserves cell viability at least for short term. In the light of the above data, we can assume that ACAIN and CACAIN are attractive alternatives for labeling of tonoplasts in living cells for the above reasons: (1) they label all tonoplast-coated compartments including large vacuoles and small vesicles, (2) they are preferentially labeling the tonoplast, and (3) they preserve cell viability at least for 1 h (see Supplementary Movie 1). In addition, they are excitable at 405 nm, so they can be easily used when multiple labeling procedures involving blue (488 nm), green (543 nm), and/or red (633 nm) wavelength excitation are used simultaneously.

In the next step, we were looking for the possible use of ACAIN/CACAIN for the study of stress-induced changes of vacuolar organization. We used microcystin-LR (MCY-LR) for this purpose (see "Introduction" section for reasons). This toxin is widely used as a tool for the regulation of structural organization and dynamics of subcellular compartments (Máthé et al. 2016). Vacuolar organization including the formation of lytic vacuoles and autophagosomes is sensitive to biotic and abiotic stresses including oxidative stress (Liu and Bassham 2012). ACAIN/CACAIN labeling revealed that MCY-LR, known to be a potent inhibitor of serine-threonine protein phosphatases (mainly PP1 and PP2A) and a ROS inducer, induced the formation of small tonoplast-coated vesicles, several of which engulfed chloroplasts (Fig. 6c). At this stage, we cannot state unequivocally the nature of these vesicles. Our TEM studies revealed that MCY-LR induced the formation of multimembrane vesicles as well as vesicles incorporated into the large vacuoles resembling plant autophagosome morphology (Fig. 6k, Supplementary Fig. 3) as described by Gao et al. (2015) and Liu et al. (2012). Autophagosomes can be not only double membrane but multimembrane vesicles as well. The multimembrane structures (Fig. 6k) were similar to autophagosomes in pathogen-infected *Nicotiana benthamiana* cells, as described by Liu et al. (2005). Autophagosomes are known to fuse with vacuoles, where their contents are degraded (Liu and Bassham 2012). The formation of multimembrane vesicles detected by our TEM studies could be the result of altered lytic vacuole formation in *Arabidopsis* as well (Feraru et al. 2010).

If the multimembrane vesicles detected are autophagosomes, they are possibly not identical to the small vesicles detected in GFP-TIP2;1 plants or by ACAIN labeling. The presence of those small vesicles may indicate two distinct phenomena. The first one is the alteration of vacuole biogenesis similar to that shown in *Arabidopsis pat* mutants (Zwiewka et al. 2011). Tonoplast proteins may be present in the membrane systems of prevacuolar compartments (PVCs), when membrane traffic between PVC and vacuolar compartments is inhibited (Foresti et al. 2006). The second possibility is the enhanced lytic vacuole formation. To our knowledge, TIP3;1 and not TIP2;1 is associated to membranes of autophagosomes (Moriyasu et al. 2003). On the other hand, vesicles observed in this work are clearly delimited by tonoplasts. TIP2;1 used in our study as one of the tonoplast markers is known to be localized in both lytic and storage vacuoles (Martinoia et al. 2000).

GFP-TIP2;1 signals showed MCY-LR-induced alterations of vacuolar organization, similar to those shown by ACAIN/CACAIN labeling (Fig. 5a–e). MCY-LR (an inhibitor of both PP1 and PP2A) induced more pronounced vacuolar alterations than OA (inhibits preferentially PP2A) and Tm (PP1) (Fig. 6f–i). Therefore, probably both PP1 and PP2A play an

important role in the differentiation of a normal vacuolar system. It is worth mentioning that to date, there is practically no information on how protein phosphatase inhibitors influence plant endomembrane integrity. For vertebrate cells, it is known that MCY-LR induces ER stress, mitochondrial damage, and autophagy (Chen and Xie 2016). Thus, our new fluorochromes ACAIN and CACAIN are presumably suitable for the detection of multiple vacuole types and stress-induced vacuolar changes. Taken together, the ACAIN/CACAIN labeling and TEM studies, the protein phosphatase inhibitor MCY-LR induces probably two types of changes: (1) alteration of vacuolar organization and (2) the formation of autophagosome-like structures.

In conclusion, ACAIN and CACAIN can be of universal use for the preferential labeling of tonoplasts in living plant cells and the study of tonoplast dynamics. Therefore, they may be good alternatives to the use of tonoplast-specific GFP-fusion proteins widely used for this purpose. These fluorochromes are suitable for the detection of stress-related changes of vacuolar organization as revealed by treatments with protein phosphatase inhibitors. In addition, even though ACAIN labeling is not restricted to a single protein with hydrophobic motifs, it is suitable for the detection and possible further identification of proteins with cysteine-rich hydrophobic motifs, among them tonoplast-specific proteins following SDS-PAGE.

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Compliance with ethical standards

Conflict of interest The authors declare there is no conflict of interest regarding the contents of this manuscript.

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