

Immunogold-labelling localization of chlorophyllase at different developmental stages of *Pachira macrocarpa* leaves

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Abstract

Background: Chlorophyllases (Chlases) are housekeeping proteins in plant cells. The dephytylating enzymes can catalyze chlorophyll (Chl) to form chlorophyllide, but the distribution of Chlases in plant cells is still an interesting debate. In this study, antibody of PmCLH2 was made and used by immunogold-labelling technique to detect the location of Chlase of *Pachira macrocarpa* (Pm) leaves at four developmental stages, including young, mature, yellowing, and senesced stages.

Results: The transmission electron microscopy results show that Chlases were comprehensively found in portions of chloroplast, such as the inner membrane of the envelope, grana, and the thylakoid membrane of the chloroplast, cytosol, and vacuoles at young, mature, and yellowing stages of Pm leaves, but not in the cell wall, plasma membrane, mitochondria, and nucleus.

Conclusions: PmChlases were mainly detected in vacuoles at the senescent stage, but a few were found in the chloroplasts. A pathway is proposed to explain the birth and death of Chl, Chlase, and chloroplasts in higher plants.

Background

Chlorophyll (Chl) is the most abundant photo-pigment in the world, being a key component of photosynthesis required for the absorption of radiant sunlight. Chl is bound to two photosystems (PSs) and their light-harvesting complexes (LHCs), and these pigment-protein complexes are located in thylakoids and grana (Tang et al. 2000). Chl degradation massively occurs during leaf senescence and fruit ripening, and the first step of degradation involves the removal of magnesium by magnesium dechelataase or in dephytylation by chlorophyllases (Chlases) (Hörtensteiner and Krautler 2011; Shimoda et al. 2016). Chlases (EC3.1.1.14) are housekeeping proteins in plant cells, and can be detected in unicellular algae and higher plants (Kermasha et al. 1992; Khalyfa et al. 1995; Hörtensteiner 1999). Chlase is thought to be the first enzyme in the Chl-degradation pathway (Matile et al. 1999). Biological functions of Chlases in Chl degradation and cell damage were reported by Kariola et al. (2005), and many reports have indicated that Chlases are located in chloroplasts and endoplasmic reticulum (Atsushi Okazawa 2006; Azoulay-Shemer et al. 2011; Azoulay Shemer et al. 2008; Chen et al. 2012).

Two Chlases, identified from two woody plants, citrus and ginkgo, were demonstrated to direct transit peptides from their synthesis site to a chloroplast target (Jacob-Wilk et al. 1999; Okazawa 2006). Chlase activity of an herb plant and barley was detected in chloroplasts (Matile et al. 1997). However, in the Arabidopsis model, Chlase-yellow fluorescent protein signal was detected in the tonoplast but not in the chloroplast (Hu et al. 2015). Two Arabidopsis Chlases (AtCLH1 and AtCLH2), located outside the chloroplast are not essential for *in vivo* Chl breakdown during leaf senescence (Schenk et al. 2007). In some species, Chlases were predicted to be located in the chloroplast, whereas in other species there are alternative pathways of Chl breakdown operating outside the plastid (Takamiya et al. 2000), or the

involvement of Chlases in Chl breakdown is questioned (Hörtensteiner 2006). Thus, the efforts to determine the intracellular localization of Chlases have remained controversial.

Leaf senescence generally induces alterations in the structure and function of the chloroplasts, resulting in the reduction of photosynthetic activity, the amount of Chl, the light absorbing capacity, and the photochemical activity of PSII (Humbeck et al. 1996). Previously, we revealed that leaves of *Pachira macrocarpa* (Pm) contained significantly higher Chlase activity than the leaves of other tested plant species, and Chlases in these Pm plants exhibited higher substrate preference toward the Chl b than Chl a (Chen et al. 2012). In addition, Western blot analysis after separation of the thylakoid membrane and the inner envelope also confirmed that PmCLH1 was located in the chloroplast inner membrane (Chen et al. 2012). The combination of amino acid sequence analysis and the subcellular localization of PmCLHs predicted by using four Web-based prediction programs show that PmCLH1 was located in chloroplasts, and PmCLH2 was a cytoplasmic and chloroplast protein (Chen et al. 2014). In this study, an immunogold-labelling technique was used to study the subcellular localization of Chlases at four different developmental stages of *P. macrocarpa* leaves, and a model of the transport route of Chlase in higher plants is proposed.

Material And Methods

Plant material and growth

Commercial *P. macrocarpa* plants were purchased, transplanted into soil medium consisting of peat moss, loamy soil, and sand in a ratio of 2:1:1, and cultured in a controlled-environment greenhouse maintained at 25/20 °C day/night temperature at 60% relative humidity. Plants were watered every other day, and an optimal amount of compound fertilizer solution (N-P₂O₅-K₂O, 20-20-20) was applied once a week. The leaves were cut, separated by stage, and used for the following analysis.

Reflective spectrometer

Flat leaf within each developmental stage was used for measuring spectral reflectance within the visible and near-infrared regions (PolyPen RP400, Photon Systems Instruments, Drasov, Czech Republic). The spectral mode of 256 bands in the 400 – 790 nm range were used to calculate normalized difference vegetation index (NDVI) and photochemical reflectance index (PRI) values.

Leaf content of Chl and flavonoid (Fla)

The leaf epidermal Chl content was obtained by measuring light transmission at 710 nm and in the near-infrared at 850 nm (Dualex Scientific, Force-A, Orsay, France). The Chl Dualex index was given by the formula: $CHL = [(I_{850} - I_{0, 850}) / (I_{710} - I_{0, 710})] - 1$, where I and I_0 were the signals measured with and without the leaf sample in the leaf clip, respectively (Cerovic et al. 2012). Moreover, the Dualex sensor index of flavonoid (Fla) content was obtained by transmitting lights at 375 nm. Nitrogen balance index (NBI),

calculated as Chl/Fla ratio, is a plant status indicator directly correlated with massive nitrogen content taken up by the plant leaves.

Chlorophyll fluorescence

Chlorophyll fluorescence (ChlF) emissions of four different stages of Pm leaves were monitored with a fluorescence imaging system (Imaging-PAMM-Series, MAXI version, Walz, Germany). The Fv/Fm values were calculated from leaves with 20 min dark adaptation, and were presented as the mean value of 5 leaves in each measurement.

Transmission electron microscopy (TEM) samples preparation and light microscope

Leaves were collected, cut into small cubes, and fixed for 4 h in 0.1% glutaraldehyde, 4% paraformaldehyde in 0.1M phosphate buffer (pH=7.1) at 25°C (Burry et al. 1992). Samples were then washed three times for 15 min each in 0.1 M phosphate buffer (pH=7.1) at 25°C. Tissues were dehydrated in a graded ethanol series, and embedded in LR White, and polymerized at 60°C for 12 h. Cross sections (0.8 µm thick) of fixed samples were stained with Toluidine Blue O (Merck, Darmstadt, Germany) observed under light microscope, ultrathin gold sections were cut on a Reichert-Jung ultramicrotome (Ultracut E, Leica, Vienna, Austria), collected on formvar-coated grids.

The affinity purified polyclonal antibody anti-PmCLH2 was used for immune-cytochemistry on four stages of Pm leaves. The polyclonal antibody preparation was generated in rabbits (Chen et al. 2014), diluted 1:1000, and immune-labeling was performed using Donkey anti Rabbit antibody coated on 18 nm golden particles (Jackson Immuno Research Inc., West Grove, PA, USA). The samples were then stained with 5% uranyl acetate in 100% H₂O, and post-stained with 0.5% lead citrate. The cross sections were viewed under TEM (Philips FEI Tecnai G2 Spirit Twin) under operation at 80 kV.

Results

Figure 1 displays the morphological characteristics of cross sections of palisade cells at four stages of *P. macrocarpa* leaves. In young leaves, palisade cells appeared ball-shaped or short-oval shaped, but spongy cells were ball-shaped or irregular in shape, and all of these cells contained chloroplasts (panel a). Palisade cells of mature leaves were long-oval shaped, but spongy cells were ball-shaped, and many chloroplasts were present in these cells of mature leaves (panel b). In yellowing leaves, palisade cells were shrunk, but spongy cells were ball-shaped or irregular in shape, and had fewer chloroplasts than mature leaves (panel c). Both palisade and spongy cells of senescent leaves were atrophied and had lost cell contents, indicating that all cells in this stage had declined (panel d).

Figures 2 a and b show the highest average values of NDVI (0.75) and PRI (0.07) at mature stage of leaves, and xanthophyll cycle activity decreased at yellowing and senescent stages of Pm leaves. NBI index values (Fig. 2c) for young and senescent leaves were similar (17 ~18) and these values were significantly lower than both the mature stage (102) and the yellowing stage (28) of leaves, suggesting

that both of the former stages were lower nitrogen required. Figure 2d demonstrates that the highest and lowest mean F_v/F_m values were detected in the mature (0.82) and senescent (0.16) stages of Pm leaves, respectively. These data imply that LHCs were disintegrated during the yellowing and senescent stages.

Immunogold-labelling was used to detect PmCLH2 locations at different developmental stages of leaves. At the young leaf stage (Fig. 3), the golden particles were labeled in thylakoids and the inner membrane of chloroplast envelope, vacuoles, and cytosol, indicating that Chlases were located in these organelles (panels a and b). Fig. 3c shows golden particles were located in disintegrated chloroplasts in vacuoles. In addition to chloroplasts, golden particles were also discovered in vesicles (Fig. 3d). In mature leaves (Fig. 4), similar to young leaves, the golden particles were located in vacuoles, cytosol, and thylakoids. It is notable that some golden particles were observed in grana stakes and the margins of the grana (panels b-d), as well as in the inner membrane of chloroplast envelope (panel b) of mature leaves. In yellowing leaves (Fig. 5), similar to mature leaves, the golden particles were located in vacuoles, cytosol, inner membrane of chloroplast envelope, and grana. Some degrading chloroplasts were lost in the envelope, but many golden particles were labeled in the thylakoids of the chloroplasts (Fig. 5c). In the senescent stage of leaves (Fig. 6), most golden particles were located in vacuoles but few were observed in chloroplasts, regardless of whether the chloroplast envelope was integrated or not. The golden particles in the senescent stage were mainly located in the vesicle membranes and vacuoles (Fig. 6f, red arrow).

In addition to Chl degraded by Chlases in intact chloroplasts, some of chloroplasts were transported into vacuoles by the autophagosomes at young stages (Fig. 7). The degradation pathway shows that normal chloroplasts (green frame in panel a, and the enlargement in panel b) contained an intact envelope, and the chloroplast envelope disintegration started at the vacuole side (red frame in panel a, and enlargement in panel c), then chloroplasts were transported into the vacuole (pink frame in panel a, and enlargement in panel d). The disintegrated chloroplasts were lost envelope and blurred thylakoid.

Table 1 summarizes the PmCLH2 subcellular locations at different developmental stages of Pm leaves. In young, mature, and yellowing stages, PmCLH2 were located in inner membrane of chloroplasts, thylakoids, cytosol, and vacuoles. However, in senescent stage, PmCLH2 was not detected in chloroplast inner membrane, implying that CLH2 might not be transported to the chloroplast, and a small number of remaining chloroplasts generated lower photosynthetic activity. The number of golden particles in subcellular locations were analyzed using the immune-labeling TEM data (Fig. 8). There were no significant differences in the numbers of golden particles found in chloroplasts and vacuoles at young, mature, and yellowing stages of Pm leaves. However, a significantly higher number of golden particles was detected in vacuoles (4.2) compared to chloroplasts (0.2) in leaves at the senescent stage.

Discussion

Leaf senescence is a developmental process with age, and its process involves an intricate and comprehensive regulation of pathways that correspond with life stages of the leaf. Chlorophyll degradation is a phenomenon of leaf senescence, and makes the leaf color turn from green to yellow

(Fig. 1). Reflective spectrometer and microscope data show that the yellowing of *P. macrocarpa* leaves is a result of chlorophyll degradation and loss of chloroplasts (Figs. 1, 2, and 5). Chlorophyll degradation or chloroplast degradation via the autophagosome pathway can be associated with photodamage, plant senescence, or vacuole rupture (Lee and Hsu 2009; Avila-Ospina et al. 2014; Izumi et al. 2017). In our study, NDVI and PRI values were not only used to monitor plant growth condition, but also could illustrate the Chl content and as an index of the xanthophyll cycle activity, respectively. NBI displayed the Chl/Fla ratio of these leaves at various stages. Furthermore, photosynthetic activity is the important function of chloroplasts, and the Fv/Fm value indicated PSII maximum activity of leaves at various stages. Although Chlases were detected in thylakoids and the inner membrane of chloroplasts at young, mature, and yellowing stages of Pm leaves, very few of Chlases were found in leaves at the senescent stage by immunogold-labelling technique (Fig. 8).

Previously, we showed that *PmCLH2* was a nuclear-encoded gene, and PmCLH2 protein was located in cytosol and chloroplasts (Chen et al. 2014). Chlase is a kind of glycoprotein (Terpstra et al. 1986), meaning that PmCLH2 can be synthesized as precursor proteins on cytosolic polysomes, glycosylated in the endoplasmic reticulum and then imported into chloroplasts. In this study, we have shown that many golden particles were observed in cytosol and others were located in chloroplasts of leaves in young, mature, and yellowing stages (Figs. 3-5). It is notable that envelope-disintegrated chloroplasts were transported into vacuoles (Fig. 3c). In addition to young leaves, chloroplast autophagy was also observed at yellowing stage of leaves (Fig. 5b), and a series of chloroplast autophagy is shown in Fig. 7, implying that the chloroplast autophagy was a turnover pathway to remove photodamaged chloroplasts (Ishida et al. 2014; Izumi et al. 2017). Presumably, the Chlases localized in vacuoles were released from degraded chloroplasts. Reports have shown that the degradation of chloroplasts within the vacuole serves as the major pathway for chloroplast protein degradation in senescing leaves (Wittenbach et al. 1982; Lin and Wittenbach 1981). In senescent leaves, almost all PmChlases were detected in vacuoles, but a few were found in chloroplasts (Figs. 6 and 8). Further research needs to be conducted to determine whether PmCLH2 cannot be transported into chloroplast or *PmCLH2* genes have been inactivated when leaves are in the senescent stage. Many golden particles were detected in grana and thylakoids of mature and yellowing leaves (Figs. 4 and 5), indicating that during these two stages PmCLH2 participated in chlorophyll degradation in the thylakoids or grana. As mentioned above, many pigment-protein complexes were located in grana, so the grana may be the location of chlorophyll degradation by Chlase.

In the study, distributions of PmChlase in plant cells were determined and a pathway is proposed to explain the birth and death of Chl, Chlase, and chloroplasts in higher plants. Figure 9 shows a possible model for the role of PmCLH2 in cell trafficking and shows that thylakoid proteins may combine with plastoglobules which are released from inner membrane. The nuclear gene *PmCLH2* is initially activated, and PmCLH2 proteins are then synthesized in cytosol. After glycosylation and folding, PmCLH2 may be transported into the vacuoles or chloroplasts, and then degraded in vacuoles. The entire “transport into chloroplast” pathway can be: PmCLH2 is released from chaperon (1), transported into chloroplast through protein translocon complexes at the chloroplast inner membrane, and truncated transit peptide of PmCLH2 (2). After PmCLH2 has been transported into the chloroplast, the transit peptides are truncated

(3). Probably, PmCLH2 is able to anchor and localize in the inner membranes (4), thylakoids (5), and grana (6). When chloroplasts are degraded in vacuoles (7), PmCLH2 is released from the envelope-disintegrated chloroplast, and proteolytic cleavage mechanism breaks down PmCLH2 into smaller peptides and amino acids (8).

Conclusions

PmCLH2 was located in cytosol, inner membrane, thylakoids, and grana of chloroplasts and vacuoles. The amount of PmCLH2 in vacuoles was similar at all stages of leaf development. However, Chlase in chloroplast was similar at young, mature, and yellowing stages, but not at the senescent stage. Thus, a possible model for the role of PmCLH2 in cell trafficking is proposed. Although Chlase was detected in inner membrane of the chloroplast, it is not currently known whether it is only binding with chloroplast translocon or located in the inner envelope of the chloroplast. In addition, the function of Chlase located in inner membrane remains unknown.

Abbreviations

Chl: chlorophyll; Chlase: chlorophyllase; Chlide: chlorophyllide; LHC: light-harvesting complex; PSII: photosystem II; TEM: transmission electronic microscope.

Declarations

Author contributions

CMY and MYH conceived and designed the study. TCL and KHL performed the research and wrote the article. CCC and THS analyzed the data. All authors have read and approved the final version of the manuscript.

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Availability of data and materials

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1. Distribution of chlorophyllase in *P. macrocarpa* leaves localized by immunogold-labelling technique at four different developmental stages.

Stages	Young leaves	Mature leaves	Yellowing leaves	Senescence leaves
Cell wall	X	X	X	X
Chloroplast inner membrane	V	V	V	X
Thylakoid membrane	V	V	V	V
Cytosol	V	V	V	V
Vacuole	V	V	V	V
Mitochondria	X	X	X	X
Nucleolus	X	X	X	X
Adjacent cell wall	X	X	X	X

V and X represent the presence or absence of chlorophyllase, respectively, based on golden particle results at different stages of leaf development.

Figures

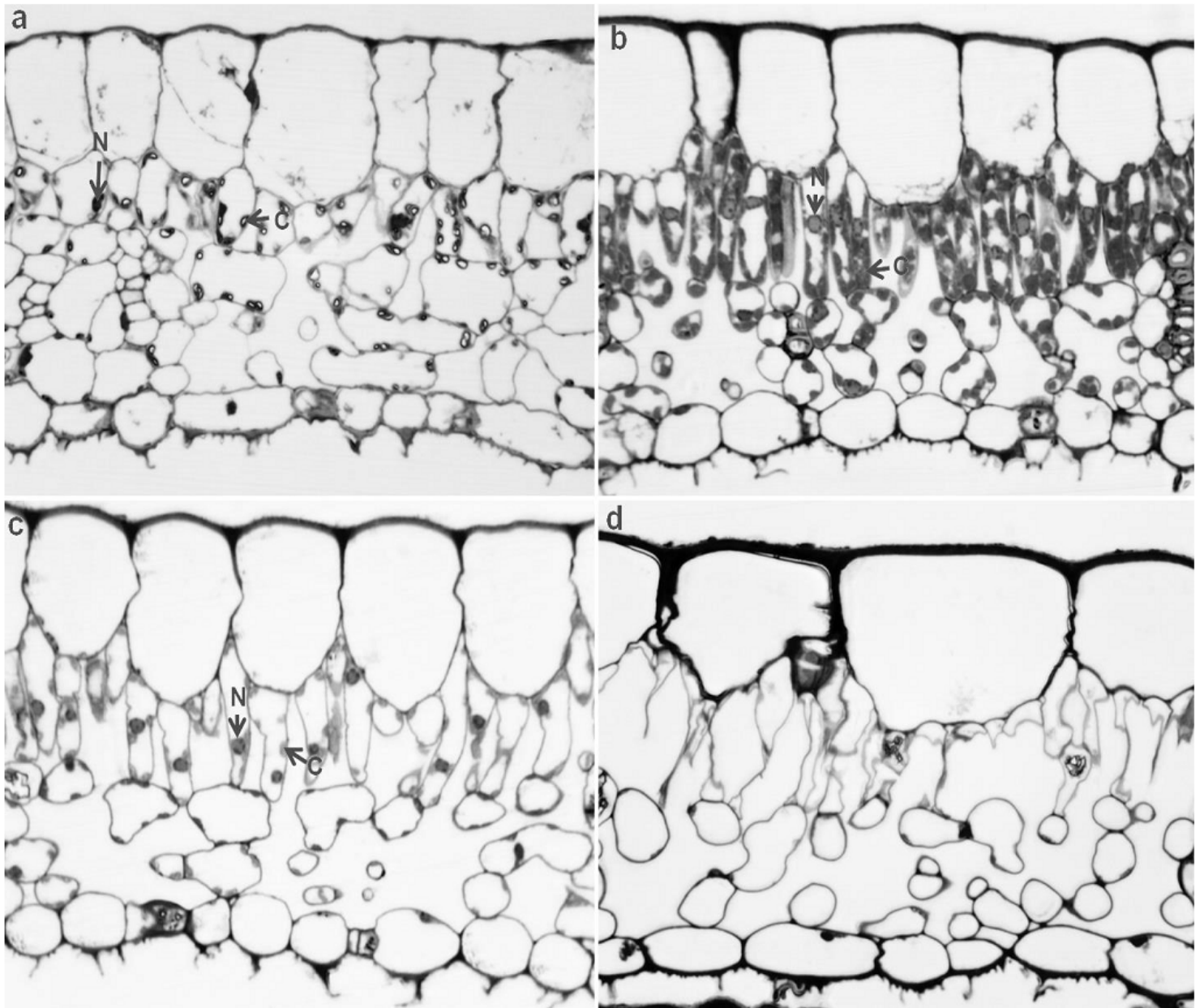


Figure 1

The cross section and morphology of *P. macrocarpa* leaves at four different developmental stages, including a young stage, b mature stage, c yellowing stage, and d senescent stage. Notes: C, chloroplast; N, nucleus

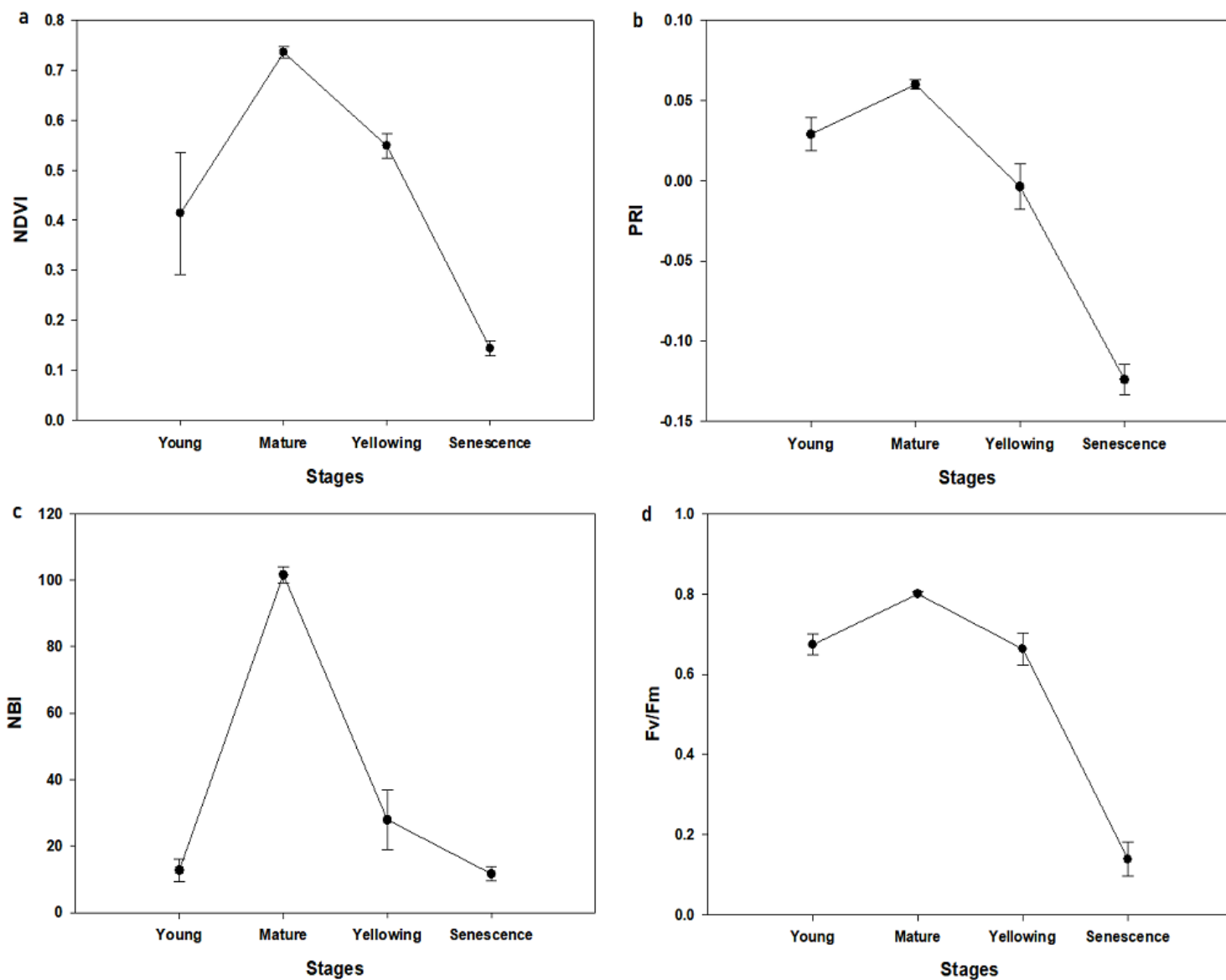


Figure 2

The parameters of NDVI (a), PRI (b), NBI (c), and Fv/Fm (d) of photosynthetic characteristics in *P. macrocarpa* leaves at four different developmental stages. Each data point represents the mean \pm standard deviation (SD) of five independent measurements with ten replicates each

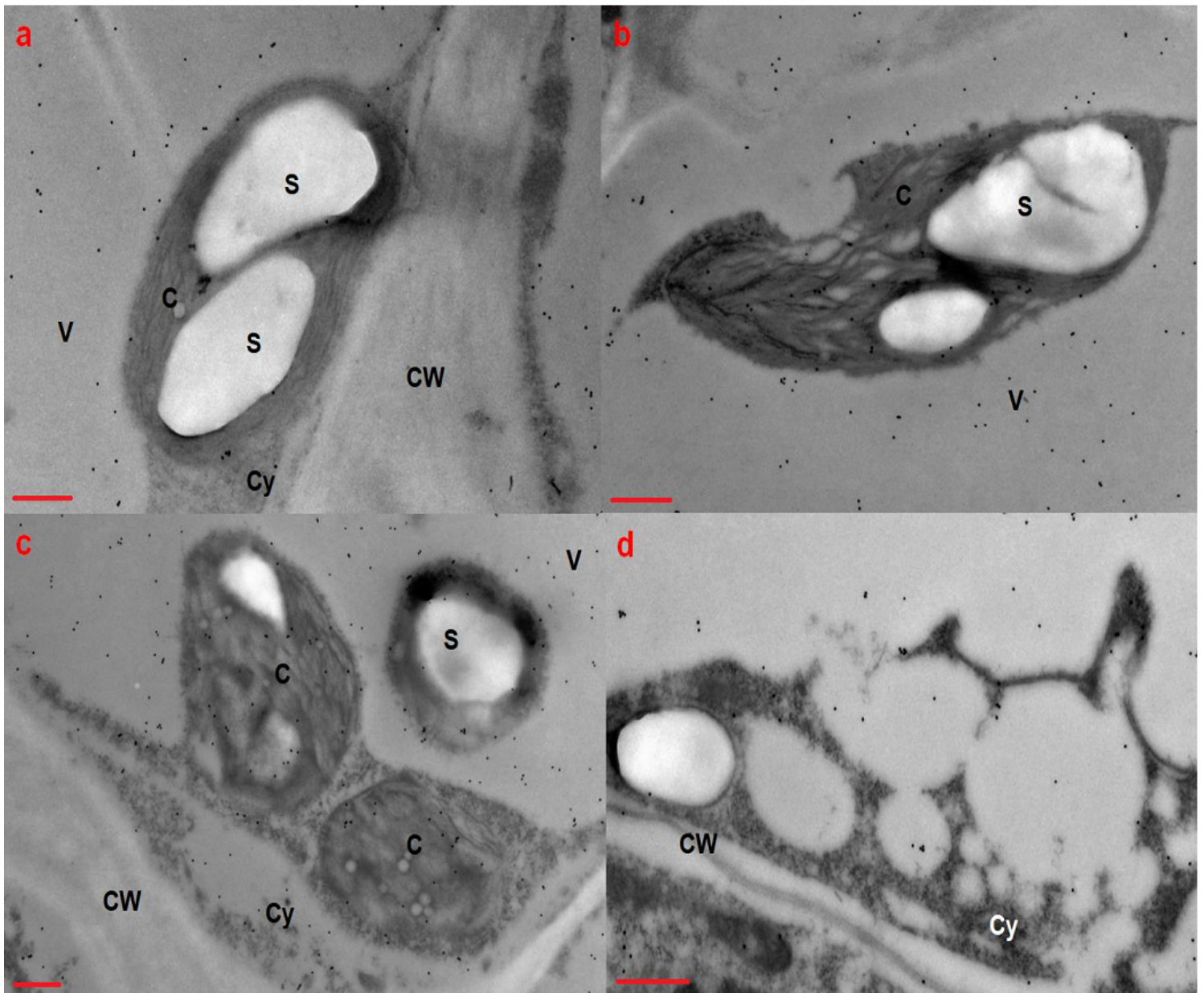


Figure 3

Immunogold-labelling distribution of chlorophyllase of *P. macrocarpa* leaves at the young stage. a A chloroplast was located in cytosol, and some golden particles were attached in inner membrane of chloroplast. b Some golden particles were located in thylakoids. c Golden particles were located in disintegrated thylakoids of the chloroplast. d Golden particles were located in vacuoles. Notes: C, chloroplast; CW, cell wall, Cy, cytosol; S, starch; V: vacuole. Bars = 0.5 μ m

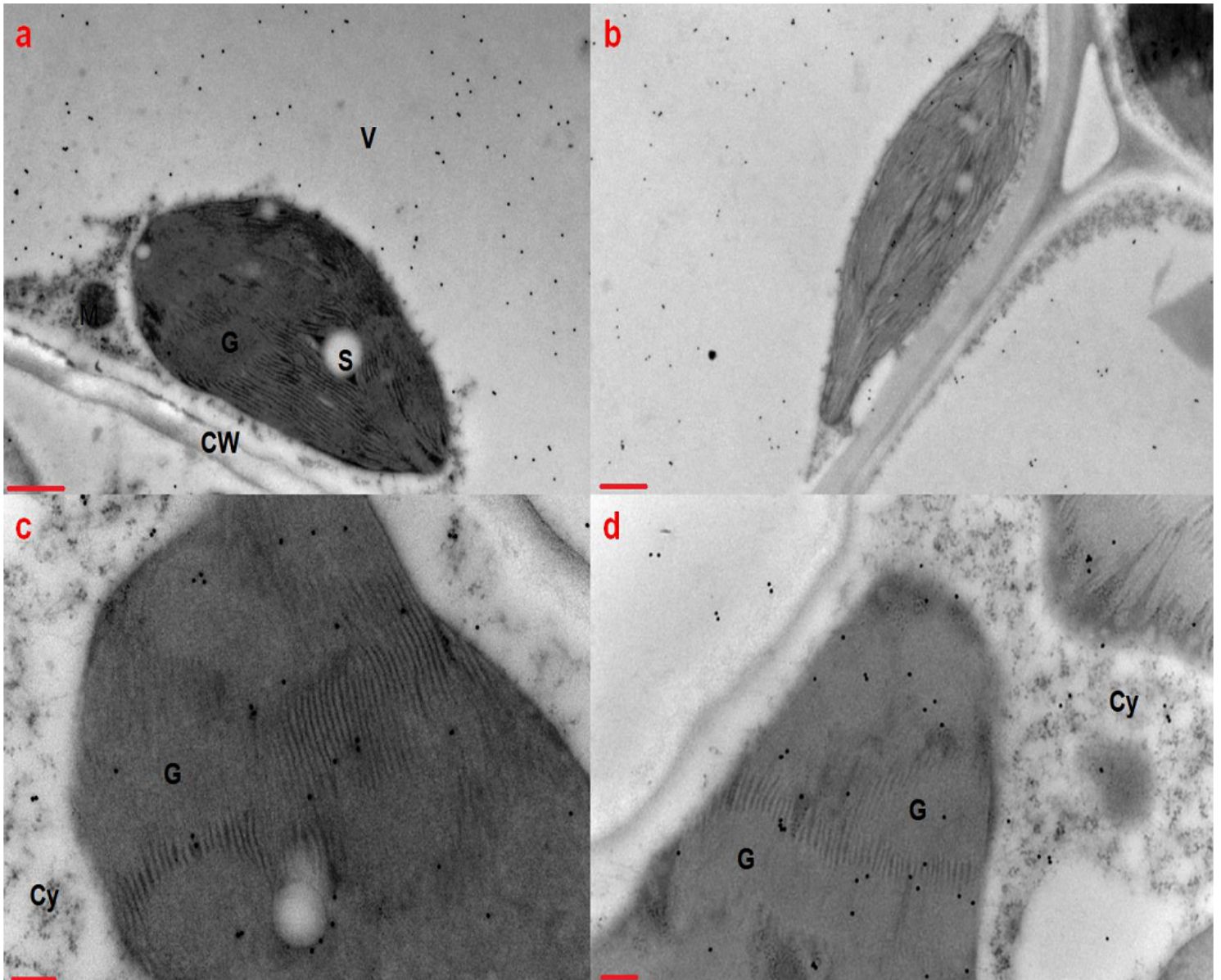


Figure 4

Immunogold-labelling distribution of chlorophyllase of *P. macrocarpa* leaves at the mature stage. a Immunogold-particles were located in the chloroplast and vacuole. b Immunogold-particles were located in the chloroplast and vacuole, but not in the cell wall and intracellular space. c Immunogold-particles were located in the thylakoids and grana, and d most of the particles were located in the margin of the grana. Notes: CW, cell wall; Cy, cytosol; G, grana; S, starch; V: vacuole. Red bars in a and b = 0.5 μm, but in c and d = 200 nm

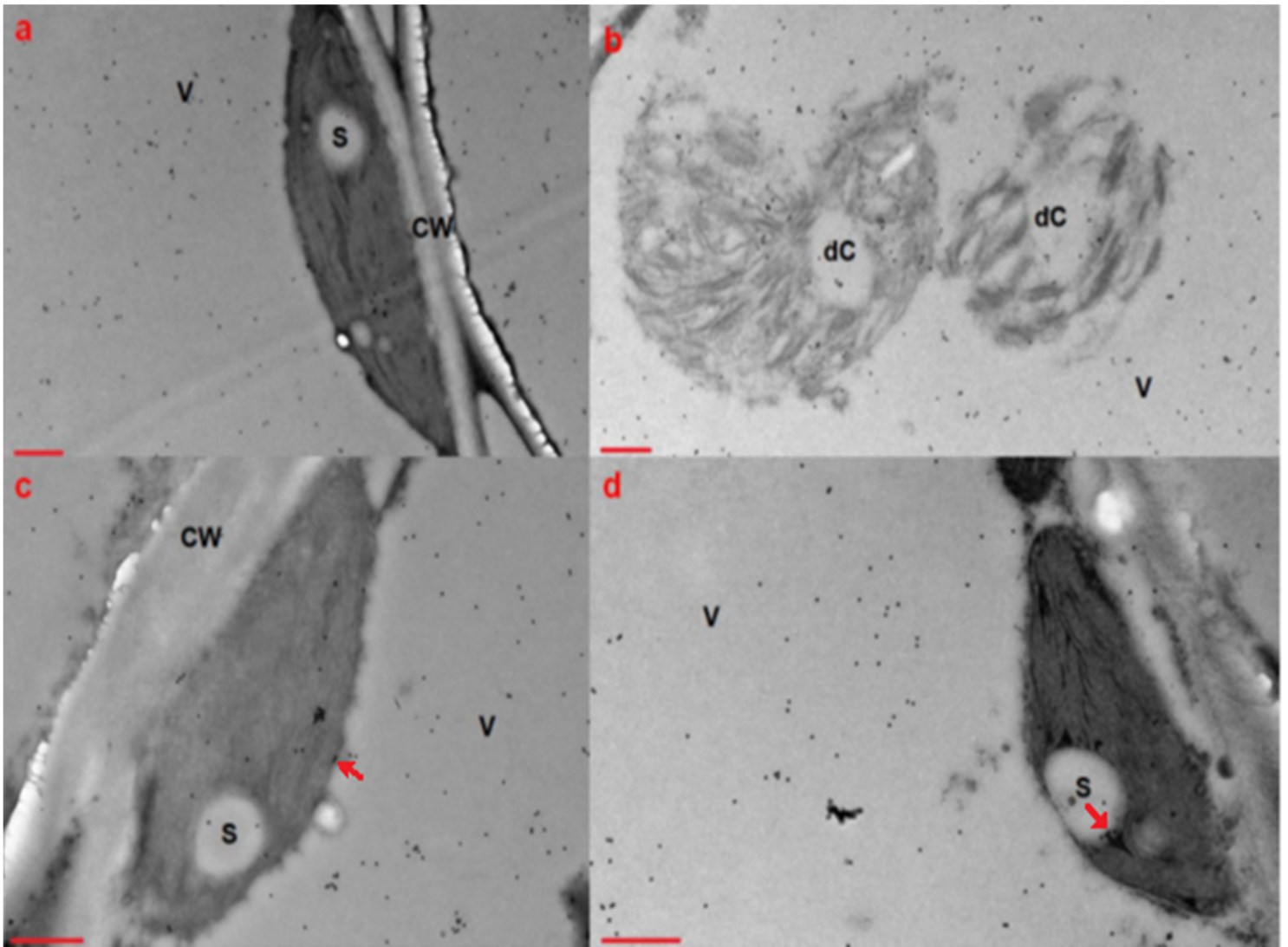


Figure 5

Immunogold-labelling distribution of chlorophyllase of *P. macrocarpa* leaves at the yellowing stage. a Immunogold-particles were located in chloroplast and vacuole, but not in cell wall and intracellular space. b Immunogold-particles were located in degrading chloroplasts. c Immunogold-particles were located in chloroplast, but some of them were located in inner membrane indicated by red arrow. d Immunogold-particles were located in the chloroplast and vacuole, but some of the particles were located in the margin of the grana and starch indicated by red arrow. Notes: CW, cell wall; dC, degrading chloroplast; S, starch; V, vacuole. Bars = 0.5 μm

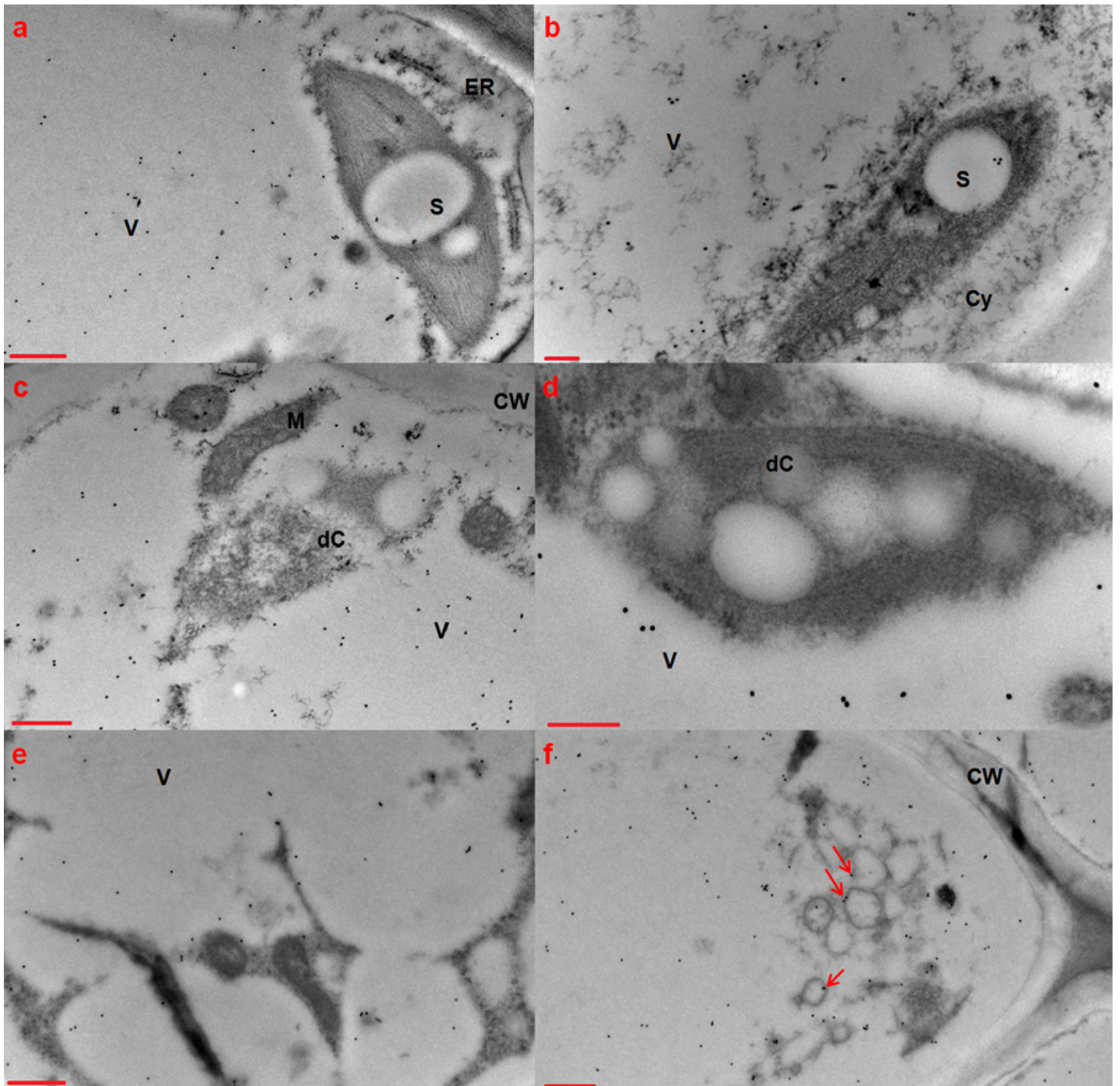


Figure 6

Immunogold-labelling distribution of chlorophyllase of *P. macrocarpa* leaves at the senescent stage. A few of (less than five) golden particles were observed in the chloroplast and disintegrated chloroplast (panels a-b), whereas most of golden particles were detected in the vacuole. No golden particles were noted in mitochondria (panel c) and disintegrated chloroplast (d). Golden particles are mainly located in the vacuole (e) and vesicle membrane (f) indicated by red arrow. Notes: CW, cell wall; Cy, cytosol; dC, disintegrated chloroplast; M, mitochondria; S, starch; V, vacuole. Bars in a, c, e, and f = 0.5 μ m, but in (b) and (d) = 200 nm

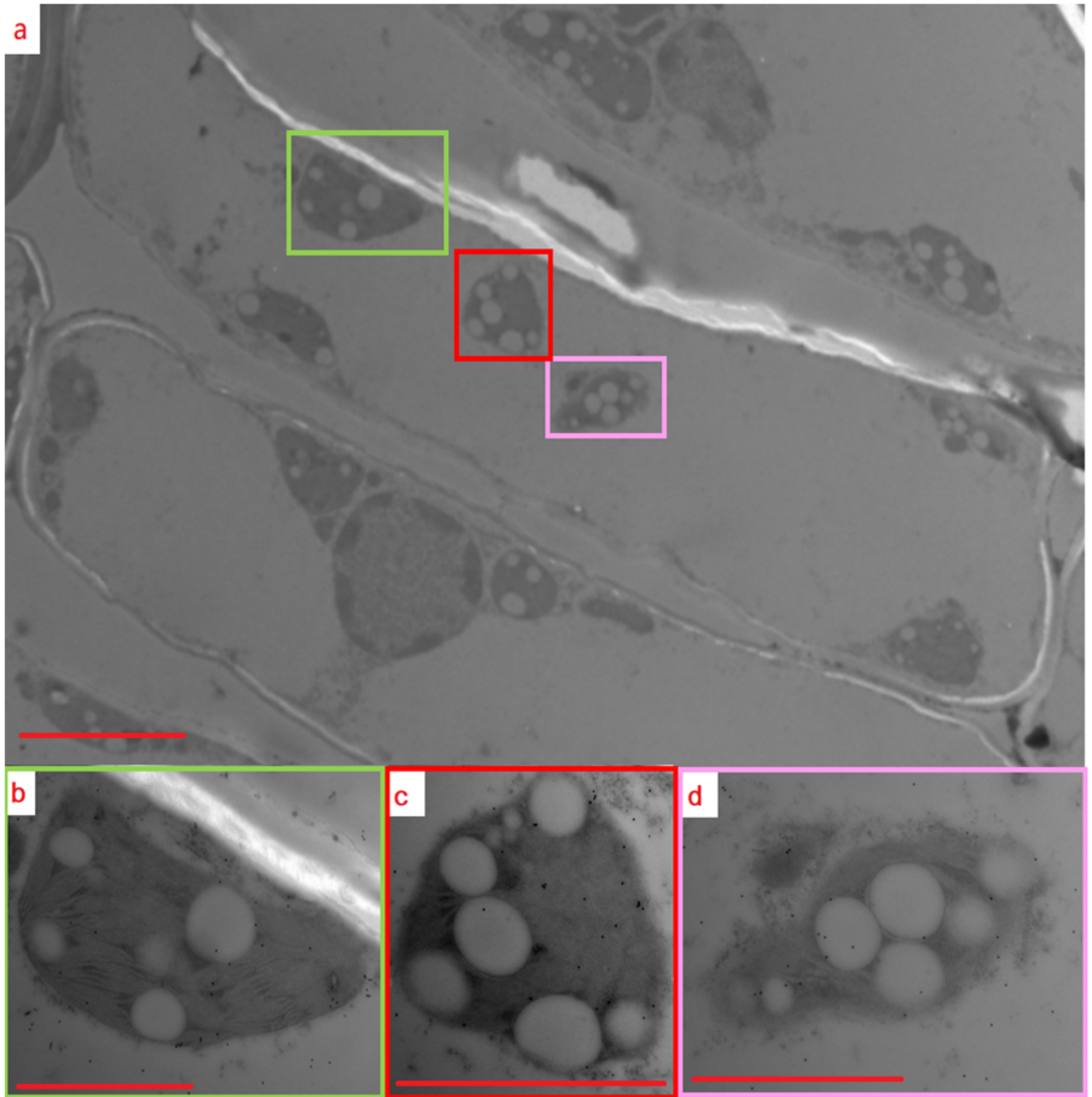


Figure 7

The autophagosome is detected during chloroplast degradation (a) of *P. macrocarpa* leaves, and the enlargement image of each color frame is shown in b-d. b Normal chloroplast, many gold-particles were detected in thylakoids. C Disintegrated envelope of chloroplast, gold-particles were observed in swelling thylakoids. d Disintegrated envelope of chloroplast, outer and inner membranes were absent, and gold-particles were noted in the vacuoles and swelling membrane system. Red bar in a = 5 μm , but in b-d = 2 μm

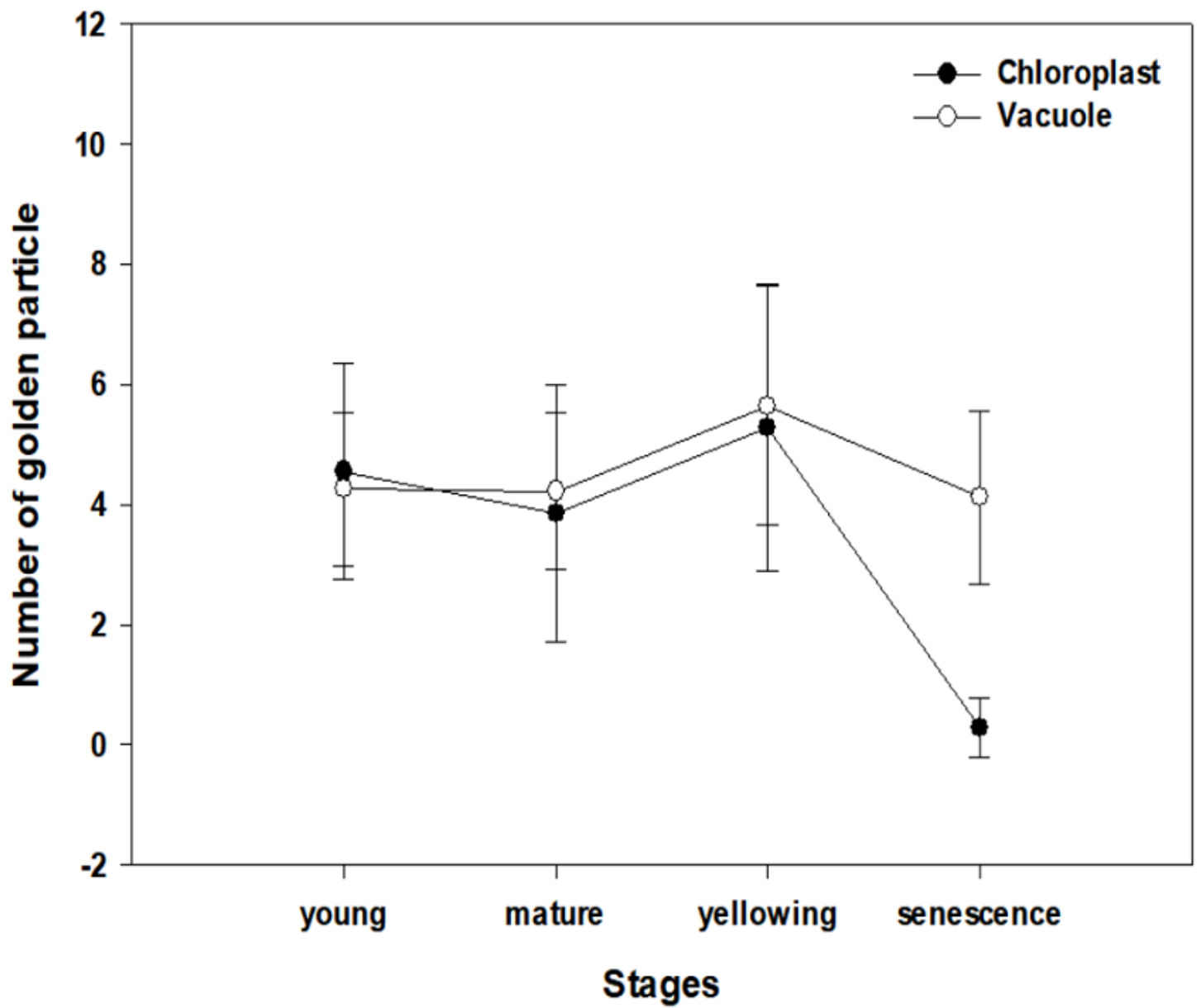


Figure 8

Number of golden particles in subcellular (per $0.25 \mu\text{m}^2$ area) localization at four developmental stages. Each data point represents the mean \pm SD of ten independent measurements

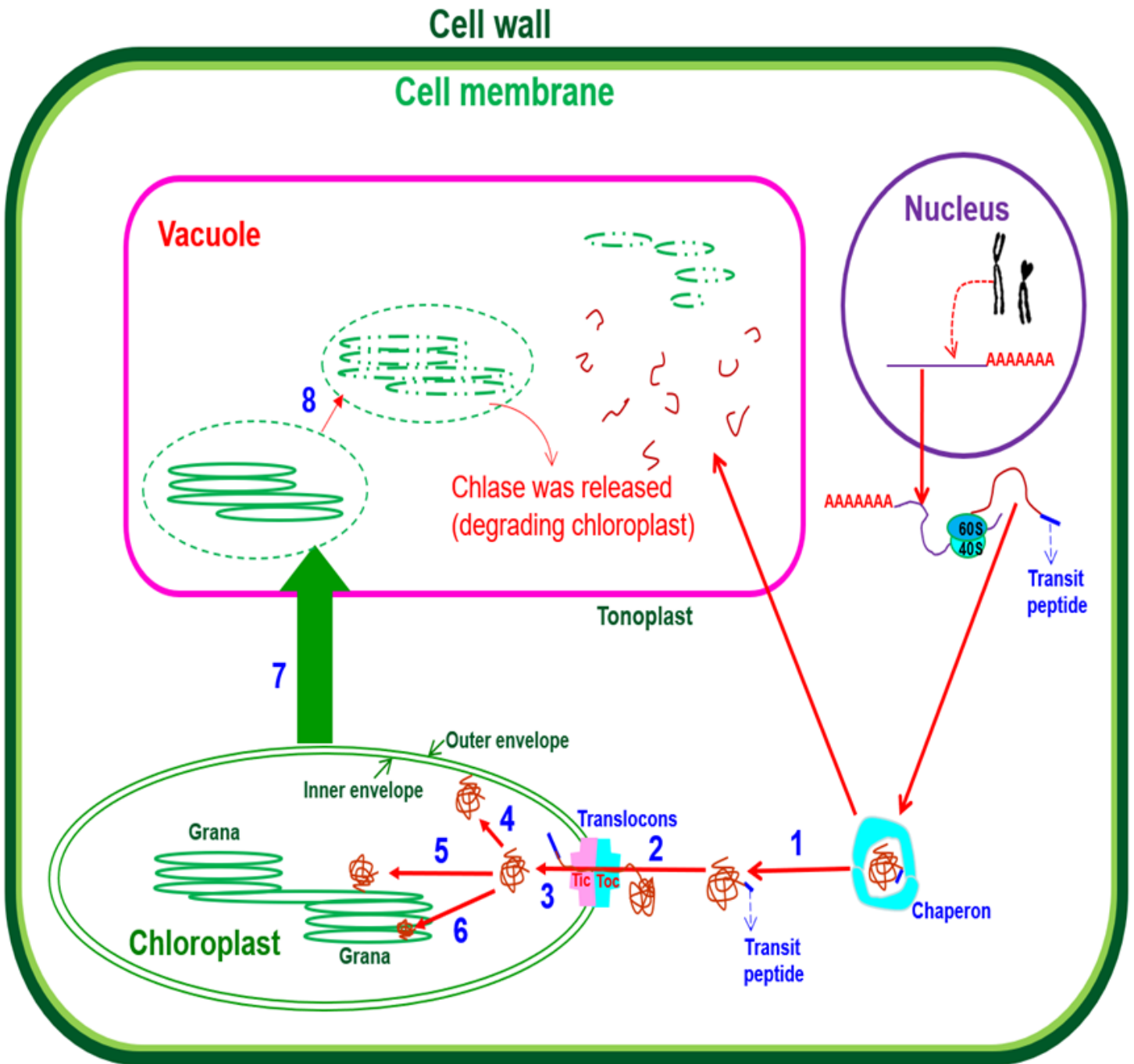


Figure 9

A simplified diagram for a possible pathway of the tour of PmCLH2 in cell trafficking. The nuclear gene PmCLH2 is initially activated, and PmCLH2 proteins are then synthesized in cytosol ribosomes. After glycosylation and folding, PmCLH2 is transported into vacuoles or chloroplasts and finally both are degraded in vacuoles. PmCLH2 could be released from chaperon (1), transported into the chloroplast through protein translocon (Toc/Tic) complexes residing at the chloroplast inner membrane. (2). After PmCLH2 has been transported into the chloroplast, transit peptides are truncated (3). It is then anchored and localized in the inner membrane (4), thylakoids (5), and grana (6). Those disintegrated chloroplasts

will be transported into vacuoles (7), degraded in vacuoles (8), and then PmCLH2 are released from the envelope-disintegrated chloroplast