

Immunogold-labelling localization of chlorophyllase-2 at different developmental stages of *Pachira macrocarpa* leaves

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Abstract

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. Previously, we showed that *PmCLH2* was a nuclear-encoded gene, and *PmCLH2* protein was located in cytosol and chloroplasts of *Pachira macrocarpa* (*Pm*). In this study, the antibody of *PmCLH2* was made and used by the immunogold-labelling technique to detect the localization of Chlase of *Pm* leaves at four developmental stages (young, mature, yellowing, and senescent). The transmission electron microscopy results show that Chlases were comprehensively found in parts of the chloroplast, such as the inner membrane of the envelope, grana, and the thylakoid membrane as well as in cytosol, and vacuoles at young, mature, and yellowing stages of *Pm* leaves, but not in the cell wall, plasma membrane, mitochondria, and nucleus. In short, *PmCLH2* was 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.

Keywords: chlorophyllase, chloroplast, immunogold-labelling, *Pachira macrocarpa*, senescence, transmission electron microscopy.

Introduction

Chlorophyll (Chl) is the most abundant plant pigment, being a key component of photosynthesis. It is required for the absorption of radiant energy by the sun. 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 chelatase 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 from unicellular algae to 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 (Okazawa *et al.* 2006, Azoulay-Shemer *et al.* 2008, 2011, Chen *et al.* 2012).

Two Chlases, identified from two woody plants, citrus,

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Abbreviations: Chl - chlorophyll; Chlase - chlorophyllase; ChlF - chlorophyll fluorescence; Fla - flavonoid; F_v/F_m - maximum fluorescence; LHC - light harvesting complex; NBI - nitrogen balance index; NDVI - normalized difference vegetation index; *Pm* - *Pachira macrocarpa*; PRI - photochemical reflectance index; PS - photosystem; ROS - reactive oxygen species; TEM - transmission electron microscopy.

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and ginkgo, were demonstrated to directly transit peptides from their chloroplast target (Jacob-Wilk *et al.* 1999, Okazawa 2006). Chlase activity of barley was detected in chloroplasts (Matile *et al.* 1997), while *Arabidopsis* Chlase-yellow fluorescent protein signal was detected in tonoplast (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). Tian *et al.* (2021) reported that AtCLH1 plays important roles in the PS II repair process, and the abundance of CLH1 peaked in young leaves and was induced by high radiation exposure. CLH1 functions in long-term adaptation of young leaves of the *var2-2 clh1-1/2-2* triple mutant to high radiation by facilitating FtsH-mediated D1 degradation *via* its Chl dephytylation activity. 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 (Hörtnersteiner 2006). Thus, the results concerning the intracellular localization of Chlases are 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 radiation absorbing capacity, and the photochemical activity of PS II (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 *Pm* leaves, and a model of the transport route of Chlase in higher plants is proposed.

Materials and methods

Plant material and growth: Commercially available plants of *Pachira macrocarpa* Walp. were purchased, transplanted into substrate consisting of peat moss, loamy soil, and sand in a ratio of 2:1:1, and cultured in a greenhouse at day/night temperatures of 25/20 °C, relative humidity of 60 %, and natural irradiance. 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 when the plants grew up to be 200 cm tall, separated by stage, and used for the following analyses.

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 was used to calculate normalized difference vegetation index (NDVI) and photochemical reflectance index (PRI) values according to Gamon *et al.* (1992). NDVI, calculated as $(R740\text{nm} - R660\text{nm}) / (R740\text{nm} + R660\text{nm})$, was used to assess the Chl content. PRI was calculated from the reflectance spectrum as $(R531\text{nm} - R570\text{nm}) / (R531\text{nm} + R570\text{nm})$ for assessing xanthophyll cycle pigments.

Leaf content of chlorophyll and flavonoids: The leaf epidermal Chl content was obtained by measuring light transmission at 710 nm and in the near-infrared region at 850 nm (*Force-A, Dualex Scientific*, Orsay, France). The Chl index was given by the formula: $\text{Chl} = [(I_{850} - I_{0, 850}) / (I_{710} - I_{0, 710})] - 1$, 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 light at 375 nm that is absorbed by flavonoids. Nitrogen balance index (NBI), calculated as Chl/Fla ratio, is a plant status indicator directly correlated with total nitrogen content in plant leaves. PRI reflects diurnal xanthophyll cycle activity.

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 variable to maximum fluorescence (F_v/F_m) values were calculated from leaves after 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 microscopy: Leaves were collected, cut into small cubes, and fixed in 0.1 % (m/v) glutaraldehyde and 4 % (m/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.1) at 25 °C for 4 h (Burry *et al.* 1992). Samples were then washed three times for 15 min in 0.1 M phosphate buffer 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) and observed under a light microscope. Then ultrathin 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 at 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*, West Grove, PA, USA). The samples were then stained

with 5 % (m/v) uranyl acetate in H₂O, and post-stained with 0.5 % (m/v) lead citrate. The cross-sections were viewed under TEM (*Philips FEI Tecnai G2*, Spirit Twin, The Netherland) under operation at 80 kV. The chloroplast of the yellowing leaves stage was used as a negative control to the specificity of the distributed labelling particles (Fig. 1 Suppl.).

In addition, absorption control of the antibody specificity was performed by mixing antibody with recombinant PmCLH2. One hundred volumes of recombinant PmCLH2 were added to the antibody and the mixture was shaken at 4 °C for 24 h. An equal volume of unabsorbed and absorbed (as negative control) antibody was applied to slides for TEM.

Results

The morphological characteristics of cross-sections of palisade cells at four stages of *P. macrocarpa* leaves are shown in Fig. 2 Suppl. In young leaves, palisade cells appeared ball-shaped or short-oval shaped, but spongy cells were ball-shaped or irregular, and all of these cells contained chloroplasts. Palisade cells of mature leaves were long-oval, but spongy cells were ball-shaped, and many chloroplasts were present in the cells of mature leaves. In yellowing leaves, palisade cells were shrunk, but spongy cells were ball-shaped or irregular in shape and had fewer chloroplasts than mature leaves. Both palisade and spongy cells of senescent leaves were atrophied and had lost cell contents, indicating that all cells in this stage had declined.

The highest average values of NDVI (0.75) and PRI (0.07) were found in leaves at a mature stage of *Pm*, and

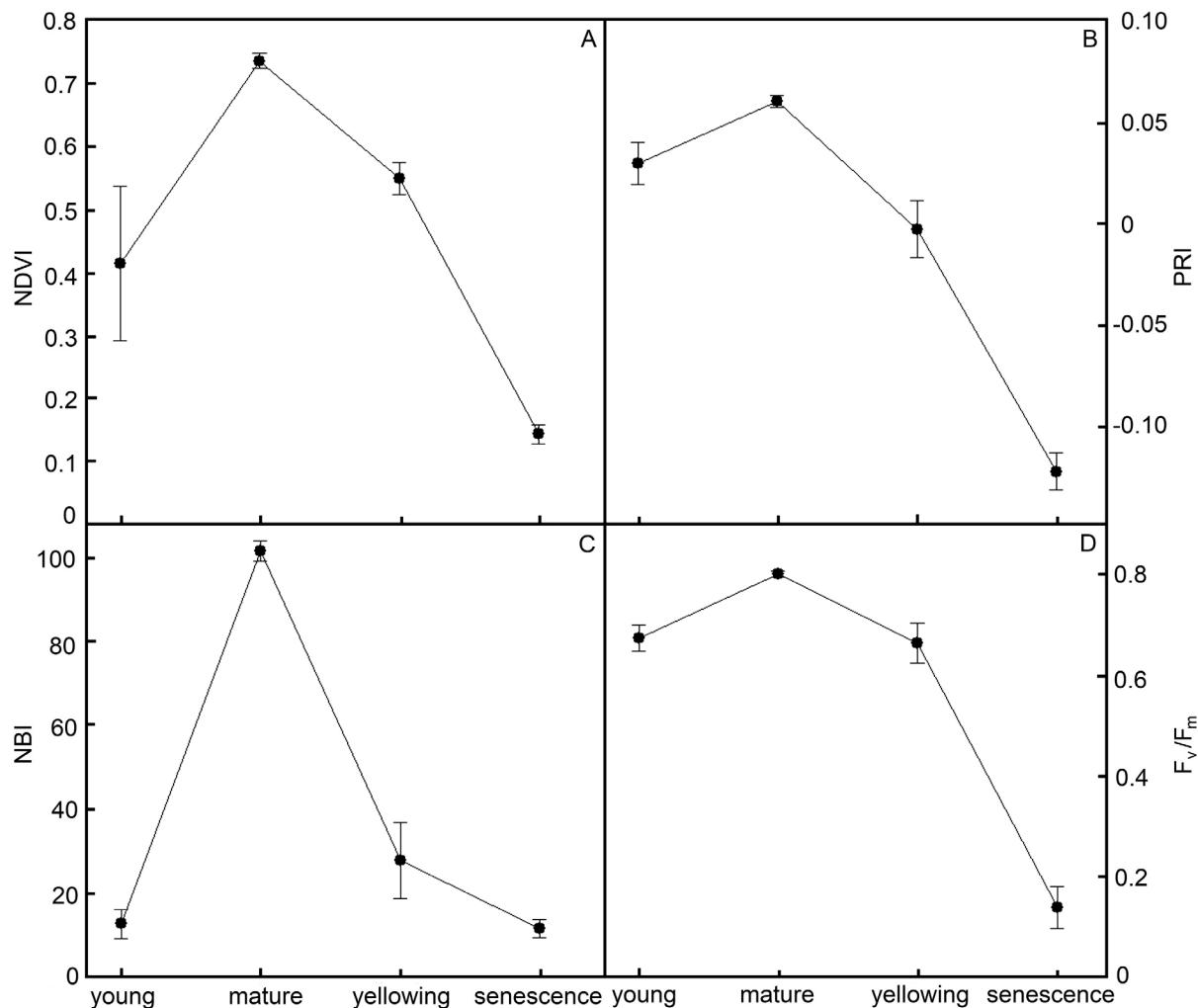


Fig. 1. The parameters of NDVI (A), PRI (B), nitrogen balance index (NBI) (C), and variable to maximum chlorophyll fluorescence (F_v/F_m) (D) as photosynthetic characteristics in *P. macrocarpa* leaves at four different developmental stages. Means \pm SDs of five independent measurements with ten replicates each. NDVI - normalized difference vegetation index, PRI - photochemical reflectance index.

xanthophyll cycle activity (detected by PRI) decreased at yellowing and senescent stages of *Pm* (Fig. 1A,B). NBI index values (Fig. 1C) for young and senescent leaves were similar (17~18) and these values were significantly lower than in the mature stage (102) and in the yellowing

stage (28) of leaves. Fig. 1D 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.

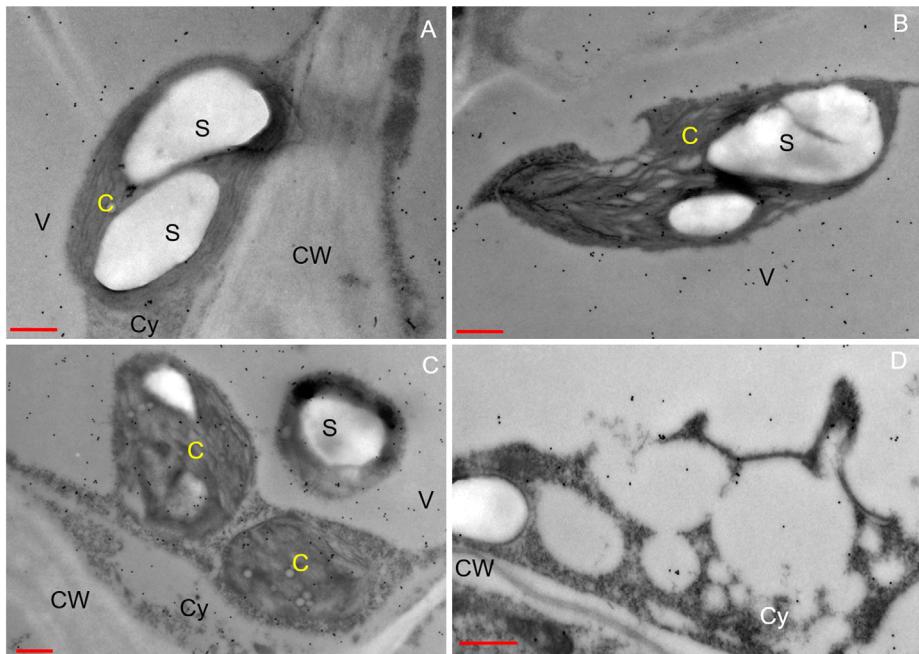


Fig. 2. Immunogold-labelling showing distribution of the PmCLH2 protein in *P. macrocarpa* leaves at the young stage. A - PmCLH2 protein is located in the cytosol, vacuole, and some golden particles are attached to the inner membrane of chloroplast; B - some golden particles are located in thylakoids; C - golden particles are located in disintegrated thylakoids of the chloroplast; D - golden particles are located in vacuoles. C - chloroplast, CW - cell wall, Cy - cytosol, S - starch, V - vacuole. Red bars = 0.5 μ m.

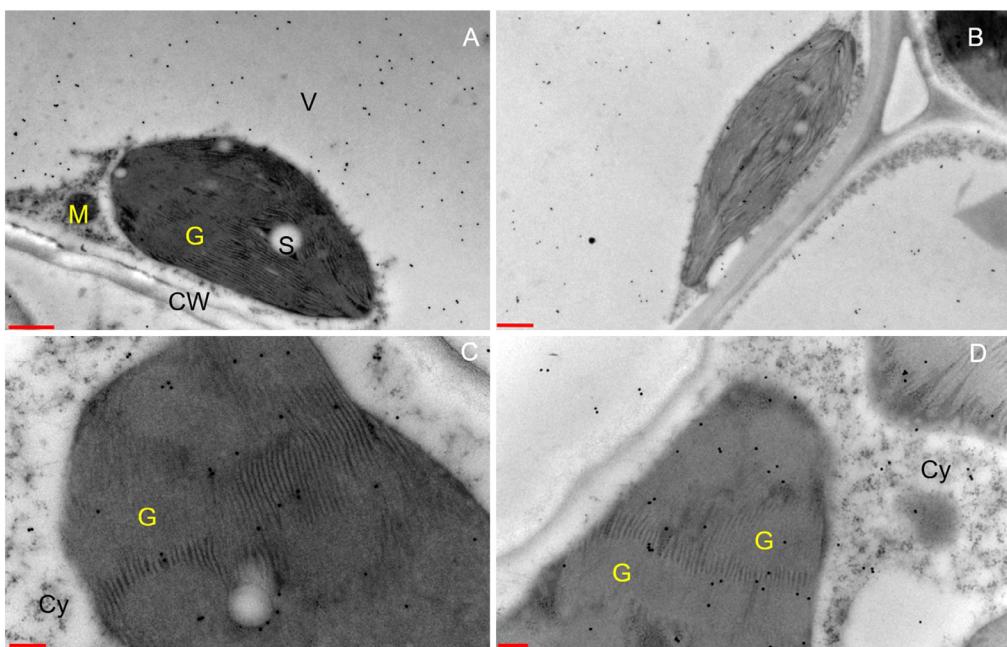


Fig. 3. Immunogold-labelling showing distribution of the PmCLH2 protein in *P. macrocarpa* leaves at the mature stage. A - Immunogold-particles are located in the chloroplast and vacuole; B - immunogold-particles are located in the chloroplast and vacuole, but not in the cell wall and intracellular space. C - immunogold-particles are located in the thylakoids and grana; D - most of the particles are located in the margin of the grana. CW - cell wall, Cy - cytosol, G - grana, M - mitochondria, S - starch, V - vacuole. Red bars in A and B = 0.5 μ m, but in C and D = 200 nm.

Immunogold-labelling was used to detect PmCLH2 locations at different developmental stages of leaves. At the young leaf stage, the golden particles were located in thylakoids and the inner membrane of chloroplast envelope, vacuoles, and cytosol indicating that Chlases were present in these organelles (Fig. 2A,B). Fig. 2C shows that golden particles were located in disintegrated chloroplasts in vacuoles. In addition to chloroplasts, golden particles also appeared in vesicles (Fig. 2D). In mature leaves (Fig. 3), similarly to young leaves, the golden particles were located in vacuoles, cytosol, and thylakoids. It is notable that some golden particles were observed in thylakoid stacks and the margins of the grana, as well as in the inner membrane of the chloroplast envelope (Fig. 3B) of mature leaves. In yellowing leaves (Fig. 4), similarly to mature leaves, the golden particles were located in vacuoles, cytosol, the inner membrane of chloroplast envelope, and grana. Some degrading chloroplasts have disrupted envelope, but many golden particles were located in the thylakoids of the chloroplasts (Fig. 4C). In the senescent leaves (Fig. 5), 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 leaves were mainly located in the vesicle membranes and vacuoles.

In addition to Chl degraded by Chlases in intact chloroplasts, some chloroplasts were transported into vacuoles by the autophagosomes at young stages (Fig. 6). The degradation pathway shows that normal chloroplasts contained an intact envelope, and the chloroplast envelope

disintegration started at the vacuole side, then chloroplasts were transported into the vacuole. The disintegrated chloroplasts lost envelope and had swollen thylakoids.

Table 1 Suppl. summarizes the PmCLH2 subcellular locations at different developmental stages of *Pm* leaves. In young, mature, and yellowing stages, PmCLH2 was located in the inner membrane of chloroplasts, thylakoids, cytosol, and vacuoles. However, in the senescent stage, PmCLH2 was not detected in the 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 numbers of golden particles in subcellular locations were analyzed using the immune-labeling TEM data (Fig. 7). There were no significant differences in the numbers of golden particles found in chloroplasts and vacuoles in 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 caused by age, and it involves an intricate and comprehensive regulation of pathways that correspond with the life stages of the leaf. Chlorophyll degradation is a phenomenon of leaf senescence, and the leaf colour turns from green to yellow. Reflective spectrometer and microscope data

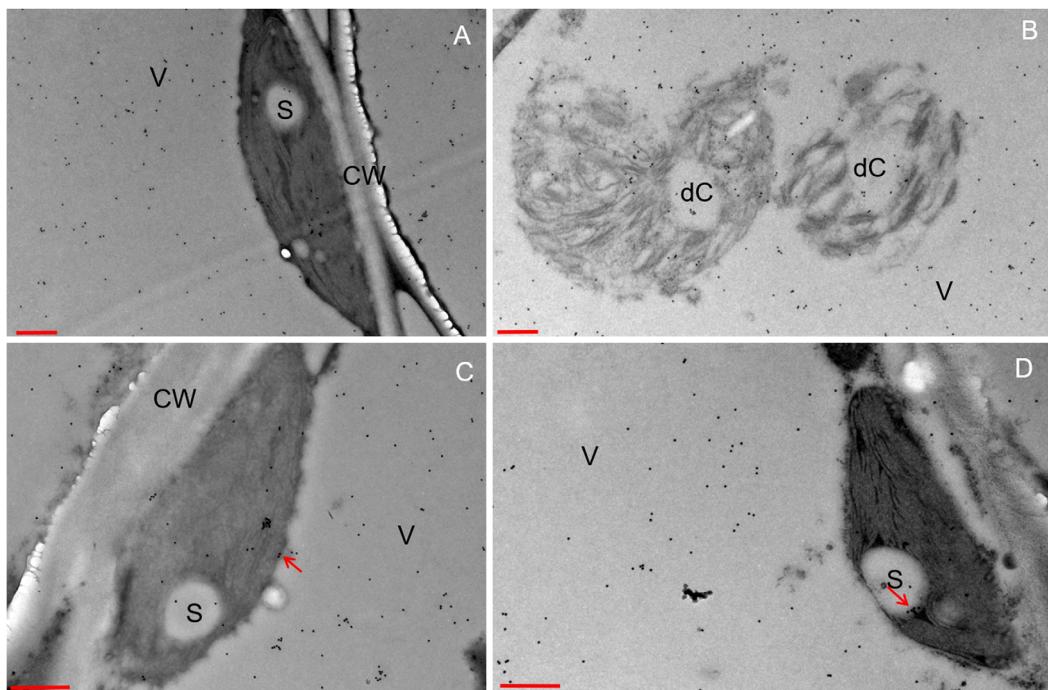


Fig. 4. Immunogold-labelling showing distribution of the PmCLH2 protein in *P. macrocarpa* leaves at the yellowing stage. A - Immunogold-particles are located in chloroplast and vacuole, but not in the cell wall and intracellular space; B - immunogold-particles are located in degrading chloroplasts; C - immunogold-particles are located in chloroplast, but some of them are located in the inner membrane as indicated by the red arrow; D - immunogold-particles are located in the chloroplast and vacuole, but some of the particles are located in the margin of the grana and starch as indicated by the red arrow. CW - cell wall, dC - degrading chloroplast, S - starch, V - vacuole. Red bars = 0.5 μ m.

show that the yellowing of *P. macrocarpa* leaves is a result of Chl degradation and loss of chloroplasts. 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). When radiation energy exceeds the capacity of the photosynthetic apparatus, such as under high irradiance, chloroplasts are damaged in a process called photoinhibition. Since this damage leads to the formation of reactive oxygen species (ROS), a reduction in the CO₂ assimilation rate, and subsequent growth inhibition, plants need to replace the damaged components within chloroplasts to maintain chloroplast function during further growth (Takahashi and Murata 2008). Direct transportation of PmCLH2 to vacuoles might be based on the presence of PmCLH2 in the vacuole at all developmental stages starting from young leaves. During the development of *P. macrocarpa* leaves, when the stage was at an earlier than young leaf stage, the high irradiance causes ROS formation in the chloroplasts and results in

damage to the chloroplast membranes. Thus, PmCLH2 in the chloroplasts would be released into the vacuoles in the young leaves. Izumi *et al.* (2017) demonstrated that exposing *Arabidopsis* leaves to high irradiance induces the vacuolar transport of whole chloroplasts *via* chlorophagy. High irradiance, *i.e.*, 2 000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, can induce ROS production and photoinhibition. Mesophyll cells in *Arabidopsis* leaves were damaged by high irradiance and they displayed abnormal chloroplasts, and these chloroplasts were enclosed by autophagosome. Thereafter, the autophagosomal structures were engulfed directly by the vacuole membrane (Nakamura *et al.* 2018).

Studies (e.g., Gamon *et al.* 1992) have shown that the reversible xanthophyll cycle can be detected by a small spectral change around 531 nm, translated to the widely used PRI, because xanthophyll cycle pigments adjust the energy distribution at the photosynthetic reaction centres. In addition, PRI has been used as a measure of photosynthetic radiation use efficiency and as stress indicator (Gamon *et al.* 1992, Peñuelas *et al.* 1995, Alonso *et al.* 2017). In our study, NDVI and PRI values were not

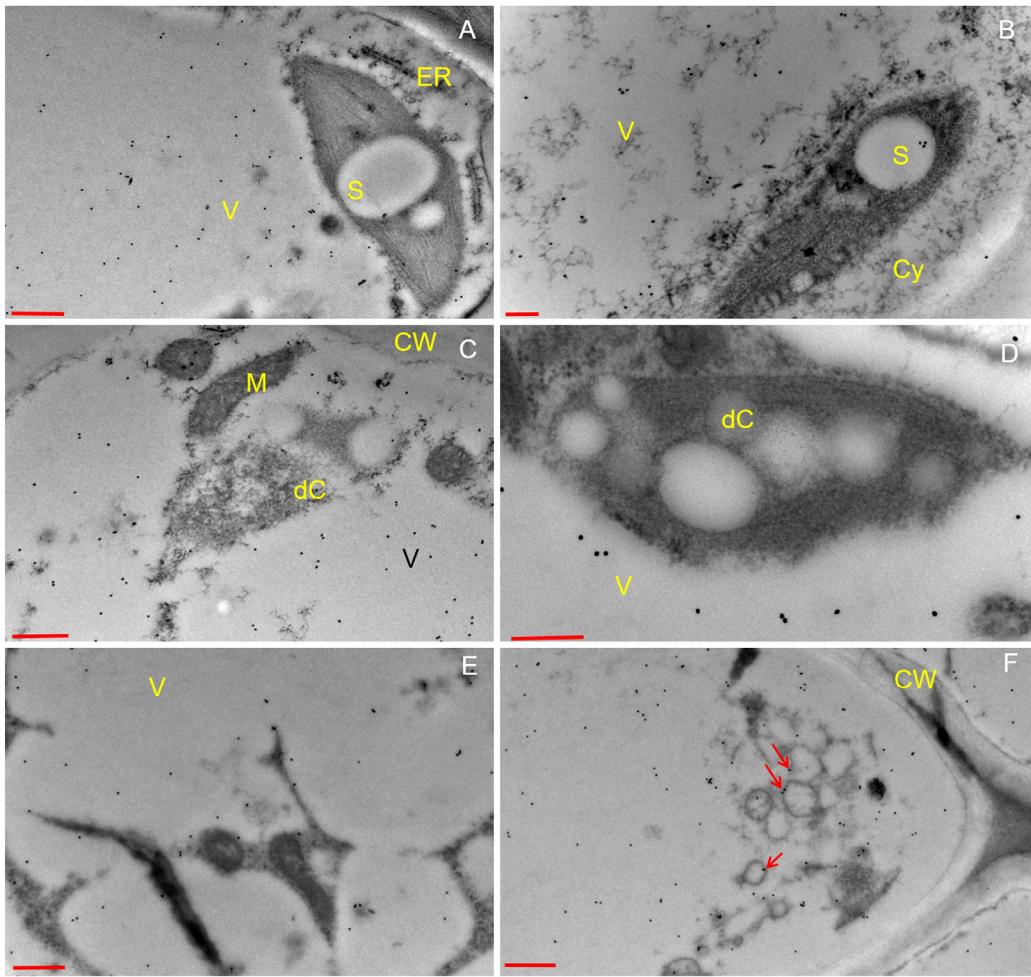


Fig. 5. Immunogold-labelling showing distribution of the PmCLH2 protein in *P. macrocarpa* leaves at the senescent stage. A few of (less than five) golden particles are in the chloroplast and disintegrated chloroplast (A, B), whereas most golden particles are in the vacuole. No golden particles are in mitochondria (C) and disintegrated chloroplast (D). Golden particles are mainly located in the vacuole (E) and vesicle membrane (F) as indicated by the red arrow. CW - cell wall, Cy - cytosol, dC - disintegrated chloroplast, M - mitochondria, S - starch, V - vacuole. Red bars in A, C, E, and F = 0.5 μm , but in B and D = 200 nm.

only used to monitor plant growth conditions but also to illustrate the Chl content and index of the xanthophyll cycle activity. NBI displayed the Chl/Fla ratio of these leaves at various stages. Furthermore, photosynthetic activity is an important function of chloroplasts, and the F_v/F_m value indicated PS II maximum activity of leaves at various stages. Although Chlases were detected in thylakoids and the inner membranes of chloroplasts at young, mature, and yellowing stages of *Pm* leaves, very few Chlases were found in leaves at the senescent stage by immunogold-labelling technique (Fig. 7). In addition, excess of nitrogen fertilizer has been found to decrease flavonoid content in fruits along with other phenolic compounds. This phenomenon has been explained by the carbon/nutrient balance hypothesis, which postulates that limited nitrogen availability leads to increased availability of carbon and carbon-based secondary metabolites (Jaakola 2013). A decrease in flavonoid content is correlated to a higher nitrogen supply, and the decrease in nitrogen supply is theoretically predictable based on the biochemical pathway leading to flavonoid synthesis (Heimler *et al.* 2017). The tomato fruits grown with the lowest nitrogen supply tend to have the highest flavonoid content (Bénard *et al.* 2009). In our study, Chl degradation can recycle nitrogen and other nutrients, and a possible explanation resides in the carbon/nutrient balance dynamic process. Flavonoid content decreased as the amount of nitrogen increased, and nitrogen deprivation was also able to increase flavonoid

accumulation in the mature *P. macrocarpa* leaves. When *P. macrocarpa* plants were grown in a nitrogen deficiency condition, Chlase activity might be increased, and nitrogen availability would be increased by decomposing chlorophyll to replenish the nitrogen source and flavonoid content would be decreased.

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.* 1981, 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. 2-4). Notably, envelope-disintegrated chloroplasts were transported into vacuoles (Fig. 2C). In addition to young leaves, chloroplast autophagy was also observed at the yellowing stage of leaves. Fig. 4B shows that *PmCLH2* was expressed in the yellowing stage (in this stage much more chlorophyll was degraded by *PmCLH2*), and the chloroplast was engulfed by vacuole, resulting in inducing chloroplast swelling associated with envelope damage and *PmCLH2* released to the vacuole. However, in the senescent stage, chloroplasts contained disorganized thylakoid membranes (Fig. 5), *PmCLH2* expression was decreased in chloroplasts in the senescent

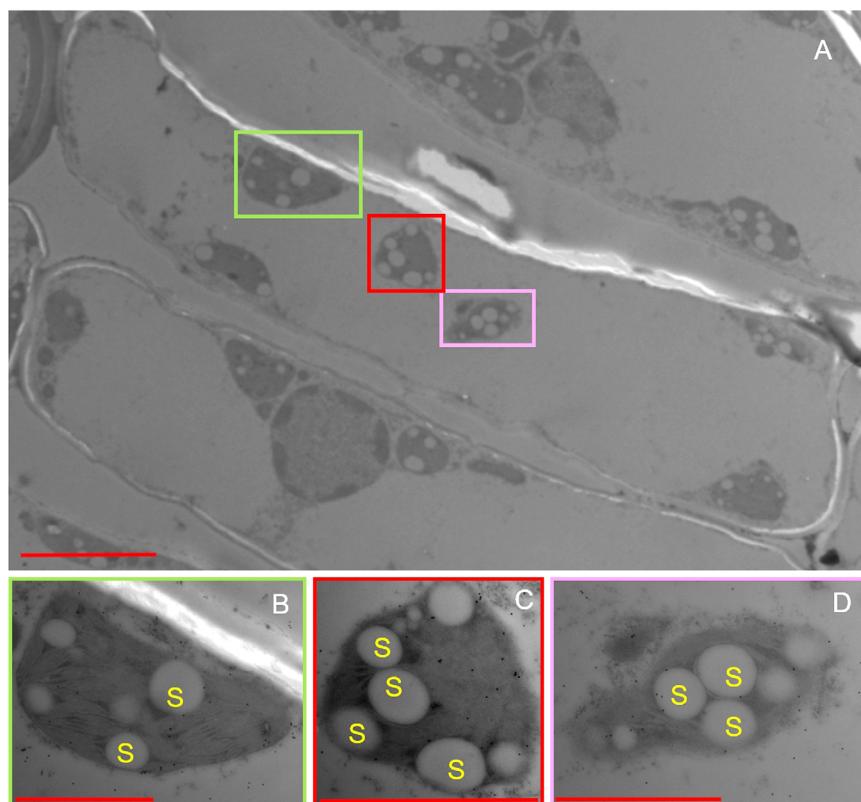


Fig. 6. The autophagosome was detected during chloroplast degradation of *P. macrocarpa* leaves (A), and the enlargement image of each color frame is shown in B - D. B - Normal chloroplast, many gold-particles are in thylakoids; C - Disintegrated envelope of chloroplast, gold-particles are in swelling thylakoids; D - disintegrated envelope of chloroplast, outer and inner membranes are absent, and gold-particles are noted in the vacuoles and swelling membrane system. S - starch. Red bar in A = 5 μ m, but in B-D = 2 μ m.

stage, and the golden particle numbers were less than in the yellowing stage (Fig. 7) and a series of chloroplast autophagy is shown on Fig. 6. These results imply 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 senescent leaves (Lin and Wittenbach 1981, Wittenbach *et al.* 1982). In senescent leaves, almost all PmChlases were detected in vacuoles, but a few were found in chloroplasts (Figs. 5 and 7). Further research needs to be conducted to determine whether PmCLH2 cannot be transported into the 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. 3 and 4), 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 this study, distributions of PmChlase in plant cells were determined and a pathway was proposed to explain the birth and death of Chl, Chlase, and chloroplasts in higher plants. Fig. 3 Suppl. shows a possible model for the role of PmCLH2 in cell trafficking and it shows that thylakoid proteins may combine with plastoglobules. The nuclear gene *PmCLH2* is initially activated, and PmCLH2 proteins are then synthesized in the 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

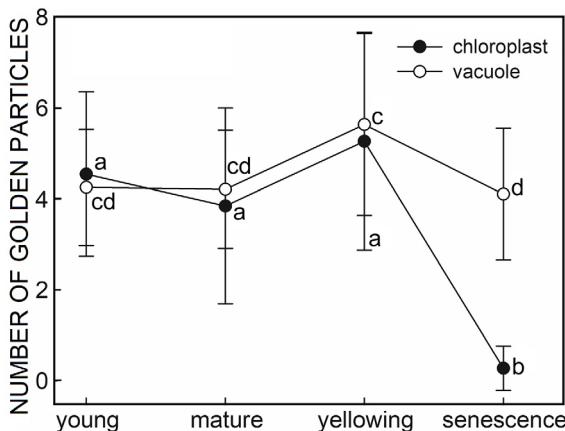


Fig. 7. Number of golden particles (per $0.25 \mu\text{m}^2$ area) in subcellular localization at four developmental stages. Means \pm SDs of ten independent measurements.

chloroplasts are degraded in vacuoles (7,8), PmCLH2 is released from the envelope-disintegrated chloroplast, and the proteolytic cleavage mechanism breaks down PmCLH2 into smaller peptides and amino acids.

In conclusion, PmCLH2 was located in the 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 the 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 the 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 the inner membrane remains unknown.

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