Characterization of Mg-dechelating substance in senescent and pre-senescent *Arabidopsis thaliana* leaves

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Abstract

The removal of Mg^{2+} is an important step in the chlorophyll degradation pathway and extracts from senescent and pre-senescent *Arabidopsis thaliana* leaves were analyzed for Mg-dechelatase activity, using chlorophyllin, an artificial derivative of the natural substrate, chlorophyllide. The optimum temperature and pH for this reaction were determined to be at approximately 50 ºC and 7.2, respectively. Mg-dechelatase activity was enhanced by addition of EDTA and inhibited by $MgCl_2$, $HgCl_2$ and reduced glutathione, indicating phenomenons such as retroinhibition by reaction products and dependence on the redox state of the mixture. Size exclusion chromatography was performed on *Arabidopsis* leaf extracts, and Mg-dechelatase activity was found in the fraction corresponding to molecular mass of about 42 kDa, which indicates that the Mg-dechelating compound in *Arabidopsis* is considerably larger than in other systems. During dark-induced senescence, the activity increased over time until reaching a maximum at day 4, and then decreased. The addition of plant growth regulators indicated that the accumulation of Mg-dechelatase was activated by ethylene and delayed by 6-benzylaminopurine.

Additional key words: chlorophyll degradation, chlorophyllin, pheophorbide.

Introduction

Leaf senescence is a highly regulated process that involves a general degradation of cellular structures and redistribution of the degradation products in different cell compartments. Many complex processes take place during leaf senescence, including distribution of nitrogen and other nutrients, dramatic increases in lipid peroxidation and membrane leakiness, dismantling of chloroplasts and other cellular organelles and degradation of macromolecules such as proteins, nucleic acids, lipids and chlorophyll (Chl) (Tanaka and Tanaka 2006, Cho *et al.* 2009, Procházková and Wilhelmová 2009). The degradation of Chls occurs simultaneously with a disorganization of the thylakoid membranes of the chloroplasts. A catabolic pathway for Chl degradation in senescent chloroplasts has been proposed, which begins with an early phase common to all plants, followed by species-specific modifications and disposal of breakdown products (Matile *et al.* 1999). Recent works on stay green mutants have revealed a gene termed *SGR* (*STAY-GREEN*) in rice, which is able to interact specifically with LHC 2 subunits and contributes to the dismantling process of Chl-binding complexes, a prerequisite for Chl degradation (Park *et al.* 2007). Once the Chl is released from the complexes, the catabolic pathway starts with the dephytylation of the Chl molecule by the action of chlorophyllase (Chlase, chlorophyll-chlorophyllide hydrolase, EC 3.1.1.14), leading to the release of chlorophyllide (Chlide). The second step of this pathway involves the enzymatic release of the central Mg^{2+} ion from Chlide producing pheophorbide (Pheide). This enzymatic step has been reported to occur in higher plants as well as in algae, although detailed knowledge about the reaction is scarce. Mg-dechelatase activity has been demonstrated by assessing Pheide accumulation *in vivo* as well as in isolated chloroplasts and chloroplast membranes, under conditions that prevent further

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Abbreviations: 6-BAP - 6-benzylaminopurine; Chl - chlorophyll; Chlide - chlorophyllide *a*; Chlin - Mg-chlorophyllin *a*; EDTA - ethylenediaminetetraacetic acid; MDS - Mg-dechelating substance; Pheide - pheophorbide *a*.
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catabolism of this reaction product (Matile *et al.* 1999). Two types of Mg-dechelating activity have been differentiated, which were either catalyzed by a protein or by a heat-stable low-molecular mass compound. The first possibility was proposed when a constitutive Mg-dechelatase enzyme was described and associated with chloroplasts membranes (Vicentini *et al.* 1995). The second option corresponds to a low-molecular mass compound named MDS (Mg-dechelating substance). In order to elucidate the inconsistency between these two activities, it has been proposed that MDS may act as a cofactor or chelator of the Mg-dechelatase enzyme, which is active on its own (Matile *et al.* 1996). However, in subsequent works this issue has been further elucidated. Shioi *et al.* (1996), working with *Chenopodium album*, detected that the high-molecular mass enzyme acts only on Mg-chlorophyllin (Chlin, a frequently used artificial substrate) but not on Chlide, the natural substrate, while MDS can dechelate Mg^{2+} from both sources. In regard to the biochemical properties of the two activities, the molecular sizes of MDS compounds differ in different plant systems; in *Chenopodium album* it has been reported to have a size lower than 400 Da but in strawberry experimental data indicates a 2 180 Da compound (Costa *et al.* 2002, Suzuki *et al.* 2005). Third in the Chl degradation pathway, the enzyme pheophorbide *a* oxygenase (PAO) breaks the porphyrin macrocycle of Pheide in a ferredoxin-dependent two-step enzymatic process releasing red chlorophyll catabolyte (RCC). This ring-opening step is crucial to the loss of green colour of Chl (Rodoni *et al.* 1997, Matile *et al.* 1999). The RCC formed by PAO is reduced by RCC reductase in a channeled reaction, producing fluorescent chlorophyll catabolytes (pFCC; Rodoni *et al.* 1997, Matile *et al.* 1999). The following steps include several enzymatic and non-enzymatic modifications, which produce non-fluorescent Chl derivates and end in the catabolytes being finally stored inside the vacuole.

Although Chl-to-Chlide hydrolysis catalyzed by Chlase has been considered to be the rate-limiting step in Chl breakdown, recent works have shown that *Arabidopsis* Chlases are not essential for *in vivo* Chl breakdown in senescent leaves (Schenk *et al.* 2007). Schelbert *et al.* (2009) reported the discovery of a novel plastid-localized enzyme that is essential for Chl breakdown during leaf senescence, named pheophytinase (PPH). In this given scenario, removal of Mg^{2+} by action of MDS is likely the first step during leaf senescence acting directly on Chl, followed by the removal of the phytol chain from pheophytin, which would be catalyzed by PPH (Schelbert *et al.* 2009).

In the last years, *Arabidopsis thaliana* has become the model organism to study different aspects of plant physiology. In this system, several genes associated to Chl catabolism have been cloned and characterized: two Chlase genes (*AtCLH1* and *AtCLH2*; Tsuchiya *et al.* 1999, Benedetti and Arruda 2002, Hörtensteiner 2006); a gene for Pheide *a* oxygenase (*AtPaO*; Pružinská *et al.* 2003) and a RCC reductase gene (Wüthrich *et al.* 2000). In the case of Mg-dechelatase, there has not been any work published in *Arabidopsis thaliana*, which raise the relevance of the study of MDS in this model plant. In the present study, we performed a biochemical characterization of the compound responsible for the Mg-dechelating activity on senescent and presenescent *Arabidopsis thaliana* leaves.

Materials and methods

Mature *Arabidopsis thaliana* L. (ecotype Col0) leaves were harvested from 35-d-old plants grown at temperature of 22 ºC, 16-h photoperiod with irradiance of 150 - 200 μmol m^{-2} s^{-1} (400 - 700 nm). Leaves in similar stages of development were used and processed immediately for senescence assays or frozen in liquid nitrogen and stored at -80 ºC for MDS extracts.

Leaves were ground in liquid nitrogen and 0.5 g of the resulting powder was mixed with 5 cm^3 100 % N,N-dimethylformamide (*Sigma*, St. Louis, USA) and centrifuged at 10 000 *g* for 10 min at 4 °C. The Chl content was measured in the supernatant according to Inskeep and Bloom (1985) by spectrophotometer (*Hitachi U2000*, Tokyo, Japan) All measurements were performed by triplicate.

Frozen leaves were homogenized with the following extraction buffer: 0.05 M NaH_2PO_4; 0.05 M Na_2HPO_4 (pH 7.2); *Triton X-100* 0.1 % (v/v); polyvinylpolypyrrolidone (PVPP) 1 % (m/v), in a proportion of 10 cm^3 extraction buffer per 1 g of tissue. The homogenate was stirred for 1 h and centrifuged at 13 200 *g* for 20 min at 4 ºC. The precipitate was discarded and the supernatant stored at -20 ºC. In order to attempt a partial purification, the supernatant was treated with solid ammonium sulfate to reach 40 % saturation. The mixture was left under stirring for 4 h at 4 ºC and centrifuged at 10 000 *g* for 20 min at 4 ºC. The pellet was discarded and the supernatant was treated again to reach 90 % saturation with ammonium sulfate. After stirring overnight at 4 ºC, the extract was centrifuged at the same conditions and the pellet resuspended in 0.05 M NaH_2PO_4/Na_2HPO_4 (pH 7.2) buffer and stored at -20 ºC.

Mg-chlorophyllin was prepared according to Vicentini *et al.* (1995). The starting material was either *Beta vulgaris* leaves, *Spinacea oleracea* leaves or *Spirulina* powder, all acquired commercially. Frozen leaves (30 g) or dry *Spirulina* powder (10 g) were homogenized with 100 cm^3 of 80 % acetone using an *Omnimixer* (*Omni International*, Waterbury, USA) and the resulting suspension was centrifuged at 13 200 *g* for 20 min (4 ºC). The supernatant was added with 20 cm^3 of petroleum ether to extract Chl. The ether phase was separated and mixed with 1 cm^3 of 30 % (m/v) KOH in methanol. Chlin was allowed to precipitate for 3 h at 4 ºC

in the dark and then centrifuged for 15 min at 7 000 *g*. The precipitate obtained was resuspended in the minimum necessary volume of 1 M tricine to reach pH 9 and stored at -20 ºC. Chlin concentration values were determined by measuring absorbance at 686 nm (*Hitachi U2000*) and using the equivalence: 0.200 units of Abs_{686} = 98 nM, according to Costa *et al*. (2002).

In order to obtain Chlide, Chl was extracted as described above. After petroleum ether extraction, a pressurized stream of nitrogen gas was directed perpendicular to the surface of the liquid, allowing evaporation of the solvent. Chl was dissolved in 100 % acetone and added to a preparation of a recombinant *Arabidopsis* Chlase. The recombinant fusion MBP-CORI1, containing the coding region of AtCHL1, a coronatine-induced protein from *Arabidopsis thaliana* with *in vitro* Chlase activity, was expressed in *Escherichia coli* BL21-lysE cells (Benedetti and Arruda 2002). Expression was carried in Luria-Bertani (Bertani 1951) medium containing 1 mM isopropylthio-β-galactoside for 4 h and purified by affinity chromatography on an amylose resin, according to the manufacturer's protocol (*New England Biolabs*, Ipswich, USA). Purified recombinant Chlase was employed to obtain Chlide *a* by enzymatic reaction from Chl *a* as described previously (Shioi *et al.* 1996, Suzuki *et al.* 2005).

The reaction mixture used for the detection of MDS activity contained 0.07 cm^3 of MDS extract; 0.02 cm^3 Chlin (to yield a final concentration of 98 nM); and 0.61 cm^3 of 0.05 M Tris-tricine buffer (pH 8.8) with 0.1 % Triton X-100. The final pH of the reaction mixture was 8.8. For assays at pH values different than 8.8, adjustments were made using HCl 2 M and adding NaCl 1 M and distilled water to equilibrate ionic strength. The effect of different compounds on Mg-dechelating activity was assayed by adding reaction mixtures with different amounts of EDTA, $MgCl_2$, KCl, $HgCl_2$, H_2O_2 and reduced L-glutathione.

In order to determine MDS activity with its natural substrate, Chlide *a*, different amounts of this compound were mixed with the MDS extract in the presence of either 0.05 M Tris-tricine buffer with 0.1 % *Triton X-100* or 0.02 M NaH_2PO_4 /Na_2HPO_4 buffer.

MDS activity was measured by following the increase in absorbance at 686 nm. Enzyme kinetics were performed at 50 ºC (unless noted otherwise) and a blank without the addition of enzyme extract was performed for each measurement, in order to assess non-enzymatic Mg^{2+} release.

A *GE XK 16/40* column packed with *Sephacryl S-100 HR* (GE) was used in an *Äkta* chromatographic system (*Amersham Biosciences*, Uppsala, Sweden). The column was equilibrated with 0.1 M NaH_2PO_4/Na_2HPO_4 (pH 7.2) buffer, which was also used as mobile phase. The sample volume applied was 1.5 cm^3, which was eluted at a flow rate of 0.6 cm^3 min^{-1} and 1 cm^3 fractions were collected. The column was calibrated using the following molecular mass markers: ribonuclease A (M_r 13 700), chymotrypsinogen A (M_r 25 000), ovalbumin (M_r 43 000), albumin (M_r 67 000) and blue dextran (Mr 2 000; *Amersham Biosciences*).

As an additional parameter to assess molecular size, crude extracts were filtered through 10 kDa cut-off filters by ultracentrifugation for 90 min at 6 000 *g*, or dialyzed using 12.4 kDa cut-off membranes against 0.05 M NaH_2PO_4/Na_2HPO_4 (pH 7.2) buffer for at least 12 h.

Leaves in similar stages of development were harvested and immediately treated by immersion in solutions of 100 g dm^{-3} 6-benzylaminopurine (*Sigma*), 100 g dm^{-3} ethephon (*Ethrel®*) or water (as control), with 0.1 % DMSO and 0.5 % *Tween 20*, for 10 min. After treatment, leaves were placed on Petri dishes and maintained in the dark at 22 ºC for 5 d. Samples were taken daily or every two days and extracts performed as described previously. Total Chl was extracted using 100 % N,N-dimethylformamide and the concentration measured according to Inskeep and Bloom (1985).

Each experiment was performed at least three times. For each assay, activity was measured at least by duplicate and the mean and standard deviation were calculated. Since the same trend was found, only results from the first experiments are shown.

Results and discussion

Mg-dechelating activity assays are based on the observation that a Mg-chlorin derived from Chl, Chlin, is converted enzymically to its respective pheo-derivative when incubated in the presence of an extract with MDS activity (Vicentini *et al.* 1995). Extracts from *Arabidopsis thaliana* rosette leaves were obtained, mixed with Chlin, incubated at 50 °C and the reaction mixture was followed spectrophotometrically. A reduction in the absorption maxima at 642 nm and a formation of a new absorbance peak at 686 nm was detected (Fig. 1), indicating the presence of Mg-dechelatase activity in the extracts. Partial purification by means of ammonium sulfate precipitation allowed an increase in activity of at least 100 %. MDS activity was detected in the 40 - 90 % ammonium sulfate fraction (data not shown), which agrees with similar studies carried on strawberry fruit (Costa *et al.* 2002). Crude extracts from pre-senescent and senescent leaves were also incubated and followed spectrophotometrically with Chlide *a*. In this case, no changes in absorption over time at different wavelengths were detected, regardless of the amount of Chlide *a* or the buffer composition employed, indicating no activity toward Chlide *a*.

The apparent Km value obtained from saturation kinetics for the Mg-dechelating reaction (Fig. 2.) was 62 nM, which indicates high affinity for the substrate employed, Chlin. The value obtained is higher than those reported for oilseed rape, 16.5 nM (Vicentini *et al.* 1995),

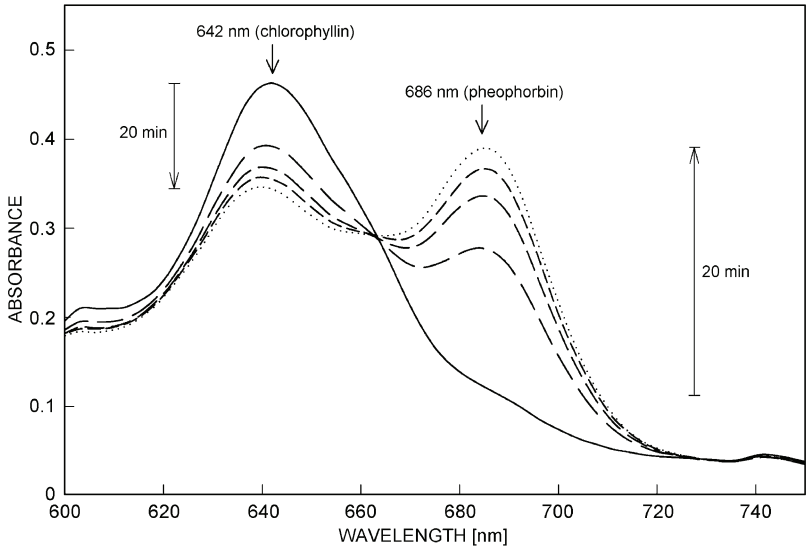

Fig. 1. Absorption spectra of the reaction mixture at different incubation times (0, 5, 10, 15 and 20 min). The decrease of the initial absorption maximum at 642 nm and the formation of a new peak at 686 nm can be seen.

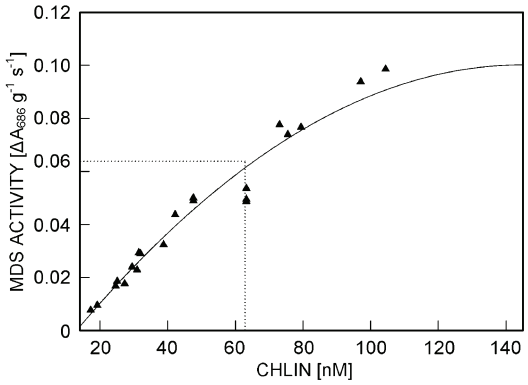

Fig. 2. Saturation kinetics for the Mg-dechelating reaction. The apparent Km value obtained is marked by the dotted line.

but lower than the values for strawberry fruit, 81 nM (Costa *et al.* 2002).

Previous works had determined that MDS activity could be associated, under certain conditions, to heat-stable low molecular mass compounds. Shioi *et al.* (1996) found that crude extracts from *Chenopodium album* lost their MDS activity after being heated at 95 ºC during 5 min. However, an extract, passed though a 5 kDa cut-off membrane and then heat treated at 95 ºC for 1 h, retained MDS activity. Moreover, in strawberry, extracts dissolved in solutions with high ionic strength lost their activity after heating at 100 °C during 10 min and could not filtrate through 10 kDa cut-off membranes; instead, extracts obtained at low ionic strength were not inactivated by the heat treatment and the activity was detected in the filtrate (Costa *et al.* 2002). Our experiments were performed in order to check if the properties of MDS from *Arabidopsis* were similar to those mentioned above. In our experiments, after filtering a crude extract through a 10 kDa cut-off membrane, MDS activity remained in the retained fraction and was not detected in the flow-through. After dialysis using 12.4 kDa cut-off tubing, activity not only remained in the residue, but appeared to increase, probably because of the loss of low molecular mass inhibitors. These results suggest that the compound responsible for MDS activity in this system could be larger than 10 kDa. In relation to heat stability, when incubating an *Arabidopsis* leaf extract at 100 ºC for over 5 min, a total loss of MDS activity was found. These results would suggest the absence of a low molecular mass compound responsible for MDS activity in *Arabidopsis*, as was previously reported in other systems, such as *Chenopodium album* (Suzuki and Shioi 2002), strawberry (Costa *et al.* 2002) and radish cotyledons (Suzuki *et al.* 2005). As reported by Suzuki *et al.* (2005), compounds with MDS activity and M_r < 3000 have substrate specificity towards both native (Chlide *a*) and artificial (Mg-chlorophyllin *a*) substrates, unlike compounds of M_r > 3000, which show evident specificity for the artificial substrate only. Thus, in the present work, no Mg-dechelating activity could be obtained when employing Chlide *a* as a substrate for activity measurements.

In order to assess the effect of pH and temperature on MDS activity, reaction mixtures were prepared at different conditions. For the optimum pH determination experiments, HCl was added to the mixture, along with appropriate amounts of NaCl and water to equilibrate ionic strength in all mixtures. Optimum temperature and pH for MDS activity were found at approximately 50 ºC and 7.2, respectively (Fig. 3). These data were similar to those reported in *Chenopodium album* (Shioi *et al.* 1996), strawberry (Costa *et al.* 2002) and *Brassica napa* (Vicentini *et al.* 1995).

Different compounds at various concentrations were added to the reaction mixture to determine their effects on

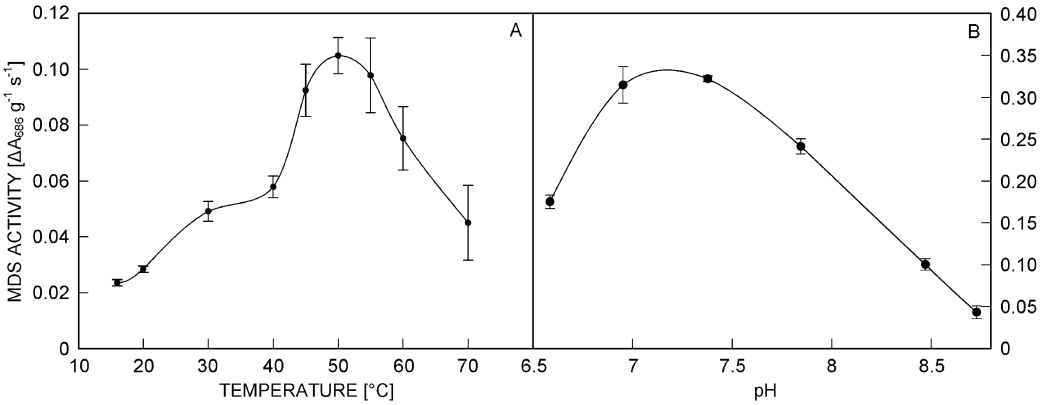

Fig. 3. Effect of temperature (*A*) and pH (*B*) on MDS activity, using Tris-tricine 0.05 M buffer. The reaction media were incubated at different temperatures and MDS activity measured, the optimum temperature was found at approximately 50 ºC. Optimum pH determination was carried at 50 ºC and using HCl 2 M, NaCl 1 M and water to adjust pH and equilibrate ionic strength.

Table 1. Effect of different compounds on MDS activity. Reaction mixtures were added at different final concentrations as described in Methods. Blanks without enzyme extract were prepared to subtract non-specific Mg^{2+} release. MDS activity values are shown as percentages relative to the respective controls without the compound added. Means ± SE, $n = 3$.

Compound	Concentration [mM]	MDS activity [%]	Compound	Concentration [mM]	MDS activity [%]
$MgCl_2$	1	93.89 ± 0.05	H_2O_2	0.1	122.39 ± 0.01
	5	75.68 ± 0.03		0.5	114.85 ± 0.00
	10	56.59 ± 0.04		1.0	109.49 ± 0.02
KCl	1	97.44 ± 0.04	glutathione	0.1	79.00 ± 0.02
	5	105.25 ± 0.03		0.5	28.31 ± 0.03
	10	103.91 ± 0.00		1.0	16.00 ± 0.00
$HgCl_2$	1	28.03 ± 0.14	EDTA	1.0	92.49 ± 0.00
	5	22.95 ± 0.05		5.0	117.47 ± 0.03
	10	21.25 ± 0.14		20.0	141.41 ± 0.06
				40.0	170.72 ± 0.03

MDS activity (Table 1). Addition of $MgCl_2$ resulted in a decrease in MDS activity, which was greater when increasing the concentration of this compound. To assess whether this effect was caused by the addition of the Mg^{2+} ion or the Cl^- ion, KCl was incorporated into the reaction mixture. Presence of KCl did not influence MDS activity, indicating that the decrease in activity with the presence $MgCl_2$ was due to the Mg^{2+} ion, a product of the reaction whose accumulation could act as inhibitor. This was further evaluated by adding EDTA, which acts as a chelating agent of Mg^{2+}. An increment of MDS activity was detected, which was also dependent on the concentration employed. The effect of these compounds is an evidence of retroinhibition by one of the end products of the MDS reaction. Inhibition of activity caused by $HgCl_2$ indicates the presence of −SH groups in the active site of this enzyme, as suggested by Vicentini *et al.* (1995). This result is complemented by the effect of reduced L-glutathione and H_2O_2 on MDS activity. An increase in activity was detected with the presence of an oxidative agent such as H_2O_2. Instead, when the reaction media was added with the reducing agent L-glutathione, MDS activity dropped considerably. The influence of the redox state in the reaction media could be related to the possible presence of -SH groups in the compound responsible for MDS activity.

A crude extract was analyzed by size exclusion chromatography using a resin with a separating range of 1 - 100 kDa and phosphate buffer as mobile phase. Only one peak of activity was detected, corresponding to the molecular size fraction of approximately 42 kDa (Fig. 4). The analysis indicates that the compound responsible for MDS activity in *Arabidopsis thaliana* is considerably larger than the corresponding compounds found in strawberry (Costa *et al.* 2002) and *Chenopodium album* (Shioi *et al.* 1996). These results, correlated with the dialysis and ultrafiltration findings mentioned before, as well as the saturation kinetics assay, suggest that in *A. thaliana*, under the experimental conditions employed, Mg-dechelating activity could be related to a relatively high molecular mass compound with enzyme-like behaviour.

To study the changes of MDS activity during senescence, rosette leaves were detached and placed in

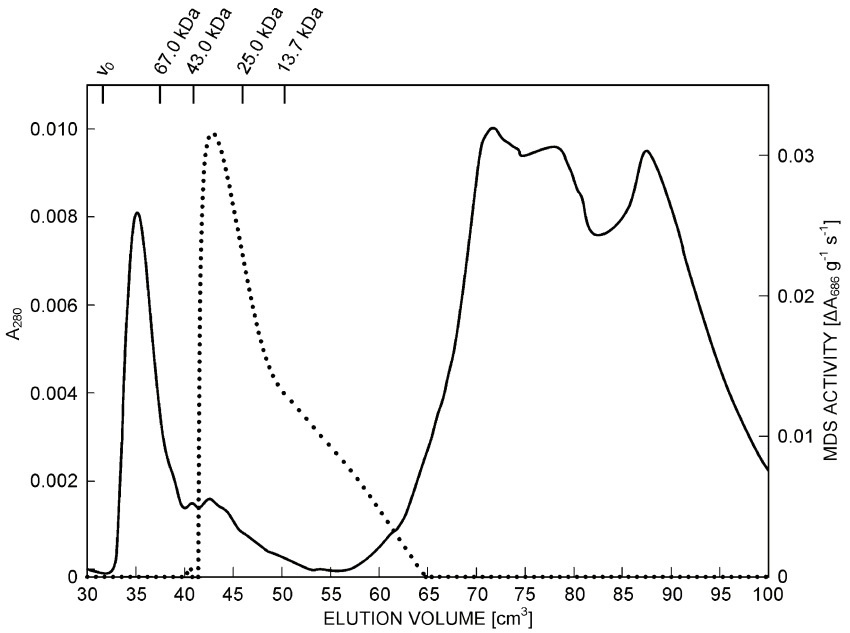

Fig. 4. Elution profile of a leaf crude extract from *Arabidopsis thaliana* obtained by size exclusion chromatography in *Sephacryl S-100 (GE)*. The absorbance at 280 nm (*full line*) was recorded and activity of each fraction (*dotted line*) was measured as described in Methods.

darkness. Leaves turned yellow and reduced their Chl content from 970 at day 0 to 190 μg g^{-1}(f.m.) at day 6. During this period, a constant increase in MDS activity was observed (Fig. 5), showing a direct relation between MDS and senescence. MDS activity was found in pre-senescent leaves and increased over the course of senescence. Research studying enzymes involved in Chl catabolism were focused mainly in Chlase and PAO, whereas Mg-dechelatase has not been studied thoroughly. A decrease of MDS activity was described during dark-induced senescence of oil-seed rape cotyledons (Vicentini *et al.* 1995). However, the activity increases during postharvest senescence of broccoli (Costa *et al.* 2005) and yellowing of *Ginkgo biloba* leaves (Tang *et al.* 2000). In the latter case, the authors suggest that this could be the key step in Chl degradation since yellow leaves showed a decrease in Chlase activity and the only catabolite detected was pheophytin. Also, in radish cotyledons, a substance named Mg-releasing protein (MRP) with activity towards Chlin and a molecular mass higher than 3 000 Da, showed an increased activity during senescence (Suzuki *et al.* 2005). Other processes with active Chl degradation such as fruit ripening also include an increment in MDS activity, as were described in strawberry (Costa *et al.* 2002) and boysenberry (Vicente *et al.* 2006).

In other set of experiments, detached leaves were treated either with ethephon (*Ethrel*®, an ethylene-releasing compound) or 6-BAP, and incubated in the dark in order to analyze the effect of these senescence regulators on MDS activity. Treatment with ethylene accelerated Chl loss, while 6-BAP delayed Chl degradation (Fig. 5). MDS activity was also affected by hormone treatments and different patterns were obtained (Fig. 5). In the case of the ethylene treatment, MDS activity values were similar to control leaves, but the increase in MDS activity due to senescence appeared to

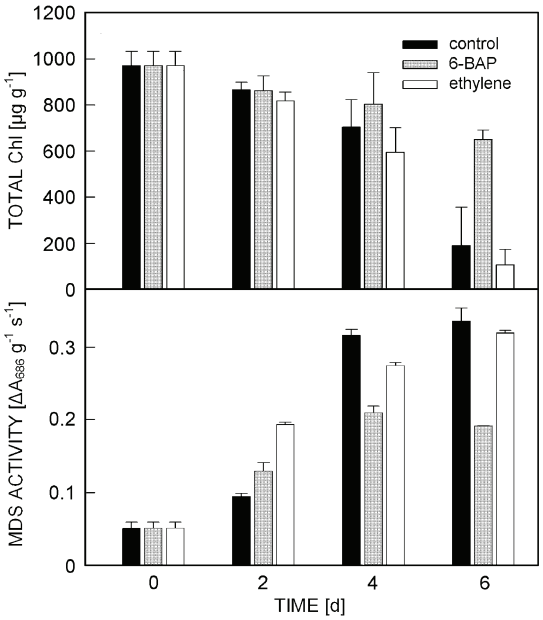

Fig. 5. Effect of 6-BAP and ethylene on MDS activity during induced senescence. Leaves were stored in darkness for 6 d and samples were taken at different periods of time after treatments with 6-BAP, ethrel or water as control. Chlorophyll content (*above*) and MDS activity (*below*) were measured at each time point.

start earlier in ethylene-treated leaves. On the other hand, in 6-BAP-treated leaves an increase in MDS activity occurred, but was lower than in control leaves. This suggests a partial inhibition of the increase of MDS activity as a consequence of cytokinin application. In broccoli florets, accelerated rates of Chl degradation and an enhance in MDS activity were observed in treatments with ethephon, while reduced rates of Chl degradation and reduced MDS activity levels were obtained in 6-BAP treated broccoli florets (Costa *et al.* 2005). Similar results were obtained by gibberellin treatments in *Paris polyphyla* leaves (Li *et al.* 2010). Moreover, increments in MDS activity during senescence of broccoli can be stalled by physical treatments (heat treatment, UV-C irradiation) that delay senescence (Costa *et al.* 2006a,b). In all these systems a clear correlation was detected between an increase in MDS activity and a decrease in Chl content. When comparing the decrease in Chl content and the increase in MDS activity over the course of the hormone treatments, the intersection point between both variables appears early in ethylene-treated leaves compared to control, indicating the accelerating effect of ethylene on plant senescence. In 6-BAP-treated leaves this intersection point appears later, probably due to the delaying effect of cytokinin over this process.

Results in the present work demonstrated the presence of MDS activity in *Arabidopsis*, determined its biochemical properties and established a correlation between the increase in MDS activity and Chl degradation during induced senescence. Unlike previous works on different plant systems, no low molecular mass compound was found with MDS activity, no substrate specificity towards Chlide was seen and the Mg-dechelating compound found shows a saturation kinetics profile similar to that of an enzyme, suggesting the presence of such compound. However, further research must be done in order to demonstrate the possible role of Mg-dechelating substance in *Arabidopsis* leaf senescence and further purification methods must be employed to determine the chemical nature of this compound.

References

Benedetti, C.E., Arruda, P.: Altering the expression of the chlorophyllase gene ATHCOR1 in transgenic *Arabidopsis* caused changes in the chlorophyll-to-chlorophyllide ratio. - Plant Physiol. **128**: 1255-1263, 2002.

Bertani, G.: Studies on lysogenesis I.: The mode of phage liberation by lysogenic *Escherichia coli*. - J. Bacteriol. **62**: 293, 1951.

Cho, C., Chung, E., Kim, K., Soh, H., Jeong, Y., Lee, S., Lee, Y., Kim, K., Chung, Y., Lee, J.: Plasma membrane localization of soybean matrix metalloproteinase differentially induced by senescence and abiotic stress. - Biol. Plant. **53**: 461-467, 2009.

Costa, M.L., Civello, P.M., Chaves, A.R., Martínez, G.A.: Characterization of Mg-dechelatase activity obtained from *Fragaria* × *ananassa* fruit. - Plant Physiol. Biochem. **40**: 111-118, 2002.

Costa, M.L., Civello, P.M., Chaves, A.R., Martínez, G.A.: Effect of ethephon and 6-benzylaminopurine on chlorophyll degrading enzymes and a peroxidase-linked chlorophyll bleaching during post-harvest senescence of broccoli (*Brassica oleracea* L.) at 20 ºC. - Postharvest Biol. Technol. **35**: 191-199, 2005.

Costa, M.L., Civello, P.M., Chaves, A.R., Martínez, G.A.: Hot air treatment decreases chlorophyll catabolism during postharvest senescence of broccoli (*Brassica oleracea* L. var. *italica*) heads. - J. Sci. Food Agr. **86**: 1125-1131, 2006a.

Costa, M.L., Vicente, A.R., Civello, P.M., Chaves, A.R., Martínez, G.A.: UV-C treatment delays postharvest senescence in broccoli florets. - Postharvest Biol. Technol. **39**: 204-210, 2006b.

Hörtensteiner, S.: Chlorophyll degradation during senescence. - Annu. Rev. Plant Biol. **57**: 55-77, 2006.

Inskeep, W.P., Bloom, P.R.: Extinction coefficients of chlorophyll *a* and b in N,N-dimethylformamide and 80 % acetone. - Plant Physiol. **77**: 483-485, 1985.

Li, J.R., Yu, K., Wei, J.R., Ma, Q., Wang, B.Q., Yu, D.: Gibberellin retards chlorophyll degradation during senescence of *Paris polyphylla*. - Biol. Plant. **54**: 395-399, 2010.

Matile, P., Hörtensteiner, S., Thomas, H.: Chlorophyll degradation. - Annu. Rev. Plant Biol. **50**: 67-95, 1999.

Matile, P., Hörtensteiner, S., Thomas, H., Kräutler, B.: Chlorophyll breakdown in senescent leaves. - Plant Physiol. **112**: 1403-1409, 1996.

Park, S.Y., Yu, J.W., Park, J.S., Li, J., Yoo, S.C., Lee, N.Y., Lee, S.K., Jeong, S.W., Hak, S.S., Koh, H.J., Jeon, J.S., Park, Y.I., Paek, N.C.: The senescence-induced staygreen protein regulates chlorophyll degradation. - Plant Cell **19**: 1649-1664, 2007.

Procházková, A., Wilhelmová, N.: Antioxidant protection during ageing and senescence in transgenic tobacco with enhanced activity of cytokinin oxidase/dehydrogenase. - Biol. Plant. **53**: 691-696, 2009.

Pružinská, A., Tanner, G., Anders, I., Roca, M., Hörtensteiner, S.: Chlorophyll breakdown: pheophorbide *a* oxygenase is a Rieske-type iron-sulfur protein, encoded by the accelerated cell death 1 gene. - Proc. nat. Acad. Sci. USA **100**: 15259-15264, 2003.

Rodoni, S., Nakayama, K., Anderl, M., Kräutler, B., Moser, D., Thomas, H., Matile, P., Hörtensteiner, S.: Chlorophyll breakdown in senescent chloroplasts: cleavage of pheophorbide a in two enzymic steps. - Plant Physiol. **115**: 669-676, 1997.

Schelbert, S., Aubry, S., Burla, B., Agne, B., Kessler, F., Krupinska, K., Hortensteiner, S.: Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in *Arabidopsis*. - Plant Cell **21**: 767-785, 2009.

Schenk, N., Schelbert, S., Kanwischer, M., Goldschmidt, E.E., Dörmann, P., Hörtensteiner, S.: The chlorophyllases AtCLH1 and AtCLH2 are not essential for senescence-related chlorophyll breakdown in *Arabidopsis thaliana*. - FEBS Lett. **581:** 5517-5525, 2007.

Shioi, Y., Tomita, N., Tsuchiya, T., Takamiya, K.I.: Conversion of chlorophyllide to pheophorbide by Mg-dechelating substance in extracts of *Chenopodium album*. - Plant Physiol. Biochem. **34:** 41-47, 1996.

Suzuki, T., Kunieda, T., Murai, F., Morioka, S., Shioi, Y.:

Mg-dechelation activity in radish cotyledons with artificial and native substrates, Mg-chlorophyllin *a* and chlorophyllide *a*. - Plant Physiol. Biochem. **43**: 459-464, 2005.

Suzuki, T., Shioi, Y.: Re-examination of Mg-dechelation reaction in the degradation of chlorophylls using chlorophyllin *a* as a substrate. - Photosynth. Res. **74**: 217-223, 2002.

Tanaka, A., Tanaka, R.: Chlorophyll metabolism. - Curr. Opin. Plant Biol. **9**: 248-255, 2006.

Tang, L., Okazawa, A., Fukusaki, E., Kobayashi, A.: Removal of magnesium by Mg-dechelatase is a major step in the chlorophyll-degrading pathway in *Ginkgo biloba* in the process of autumnal tints. - Z. Naturforsch. Sect. C **55**: 923-926, 2000.

Tsuchiya, T., Ohta, H., Okawa, K., Iwamatsu, A., Shimada, H., Masuda, T., Takamiya, K.I.: Cloning of chlorophyllase, the key enzyme in chlorophyll degradation: finding of a lipase motif and the induction by methyl jasmonate. - Proc. nat. Acad. Sci. USA **96**: 15362-15367, 1999.

Vicente, A.R., Costa, L., Covatta, F., Martínez, G.A., Chaves, A.R., Civello, P.M., Sozzi, G.O.: Physiological changes in boysenberry fruit during growth and ripening. - J. hort. Sci. Biotechnol. **81**: 525-531, 2006.

Vicentini, F., Iten, F., Matile, P.: Development of an assay for Mg-dechelatase of oilseed rape cotyledons, using chlorophyllin as the substrate. - Physiol. Plant. **94**: 57-63, 1995.

Wüthrich, K.L., Bovet, L., Hunziker, P.E., Donnison, I.S., Hörtensteiner, S.: Molecular cloning, functional expression and characterisation of RCC reductase involved in chlorophyll catabolism. - Plant J. **21**: 189-198, 2000.