BIOLOGIA PLANTARUM 46 (1): 125-127, 2003

BRIEF COMMUNICATION

Chlorophyllase activity and chlorophyll content in wild and mutant plants of *Arabidopsis thaliana*

D.T. TODOROV*, E.N. KARANOV*, A.R. SMITH** and M.A. HALL**

*Institute of Plant Physiology, Bulgarian Academy of Science, Acad. G. Bonchev Str., Bl. 21, BG-1113 Sofia, Bulgaria**
*Institute of Biological Sciences, University of Wales, Edward Liwyd Bl., SY233DA Ceredigion, Aberystwyth, UK***

Abstract

The activity of chlorophyllase in wild type (WT) was higher than in ethylene insensitive mutant (*eti 5*) of *Arabidopsis thaliana* (L.) Heynh plants during the vegetative period. Chlorophyll content in *eti 5* leaves was higher than in WT but the difference decreased by the end of the experimental period.

Additional key words: ethylene insensitive mutant, leaf age.

Chlorophyllase (Chlase) catalyzes the hydrolysis of chlorophyll (Chl) into chlorophyllide (Chlide) and phytol, suggesting a key role in Chl catabolism (Holden 1961, Matile *et al.* 1997). Chlases have been found in vegetative tissues of all ages (McFeeters *et al.* 1971, Tanaka *et al.* 1982).

Mutants of *Arabidopsis thaliana* are being used increasingly in physiological and biochemical studies (Scott 1990). In the present study we used plants of *Arabidopsis thaliana* (L.) Heynh wild type (WT) and the ethylene insensitive mutant (*eti 5*) (Harpham *et al.* 1991). The aim of the study was to establish if some known approaches for determination of Chlase activity are appropriate for *Arabidopsis* and whether there are differences between Chlase activity in wild type and *eti 5* during the vegetative period in separate leaf nodes and how far its activity is correlated with endogenous Chl content.

Seeds of wild type and the ethylene insensitive mutant of *Arabidopsis thaliana* (L.) Heynh were sown on a 2:1 mixture of soil and perlite in plastic pots in a growth chamber. The growth conditions were: day/night temperature 24/18 °C, irradiance 90 μmol m^{-2} s^{-1}, and a 12-h photoperiod. Initially, Chlase activity (EC 3.1.1.14) was determined by the methods of Holden (1961) and Trebitsh *et al.* (1993). An optimization of a reaction medium of Chlase from *Arabidopsis* plants has not been previously reported. Besides, earlier published data for a reaction medium for Chlases are different, accordingly we carried out several steps for its optimisation (data not shown). The leaves of *Arabidopsis* were homogenized in 80 % acetone at 4 °C and centrifuged at 5 000 *g* for 10 min. The pellet was resuspended in 5 mM potassium phosphate buffer (pH 7.0) containing 50 mM KCl + 0.24 % Triton X-100 and centrifuged at 12 000 *g* for 10 min. The supernatant was used for Chlase determination. Chl *a* (*Sigma-Aldrich,* Deisenhofen, Germany) dissolved in 100 % acetone was used as substrate. The assay medium contained crude enzyme extract (250 mm^3), buffer (250 mm^3) and 50 mm^3 of Chl *a* (50 μg) and it was incubated at 37 °C for 10 min. The reaction was stopped by the addition of 5.5 cm^3 of acetone : hexane : 10 mM KOH (2:3:0.5, v:v:v) and the mixture was shaken and centrifuged at 8 000 *g* for 5 min to separate the phases.

Received 24 September 2001, *accepted* 11 January 2002.
Abbreviations: Chl - chlorophyll; Chlase - chlorophyllase; DAS - days after sowing; *eti 5* - ethylene insensitive mutant; WT - wild type.
Acknowledgements: This work was supported by the COPERNICUS programme ERBIC15CT960914.
Fax: (+35) 92 739952, e-mail: dtodorov@obzor.bio21.bas.bg

Chlide *a* was determined in the acetone phase spectrophotometrically (*Spekol 11, Carl Zeiss*, Jena, Germany) at 667 nm and its concentration was calculated using an absorbance of 74.9 mmol cm^{-1} (McFeeters *et al.* 1971). Chlase activity was expressed as production of Chlide. Protein was determined by the method of Bradford (1976). Chl content was measured spectrophotometrically (Arnon 1949). The results presented are from two experiments, in four replications each determinations. The results were analysed statistically using Fisher's criteria.

Chlase activity varied substantially among leaves of different ages for both WT and *eti 5* (Fig. 1). At both 35 and 44 d after sowing (DAS) highest activity was observed at the fourth node (from the bottom) although the difference was less pronounced at 44 DAS. At 53 DAS the oldest leaves showed a marked decrease in activity and maximum activity was found at the fifth and sixth nodes. With one exception (53 DAS, third node) Chlase activity in *eti 5* was higher than in WT. In both WT and *eti 5*, Chl *a* concentration showed an increasing gradient from old to young leaves (Fig. 1). However, concentrations in *eti 5* were greater than in WT, especially in the youngest leaves at 35 and 44 DAS.

Previous reports indicate that Chlase activity varies with plant age and species (Šesták 1985). Thus, Chlase isolated from young tea, tobacco, and *Citrus* leaves was considerably higher than in older leaves (Shimizu and

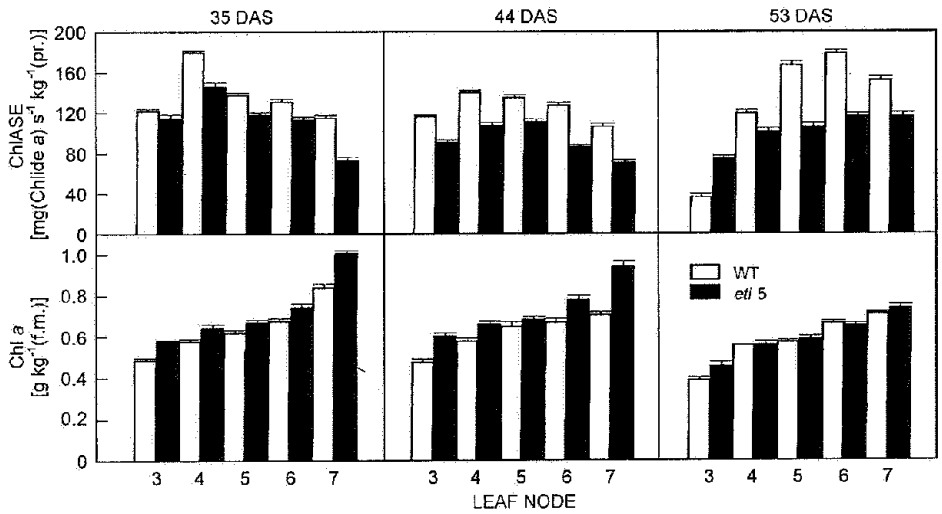

Fig. 1. Chlorophyllase (Chlase) activity and chlorophyll (Chl) *a* content in *Arabidopsis* leaf nodes during the vegetative period. LSD: 5 % = 3.167 (Chlase) or 0.017 (Chl *a*); 1 % = 4.167 (Chlase) or 0.023 (Chl *a*).

Tamaki 1962, Ogura 1969, Kuroki *et al.* 1981, Garcia and Galindo 1991). In greening oranges Chlase and Chl content increased in tandem (Aljuburi *et al.* 1979) and in olives Chlase activity was at a maximum during two periods when Chl synthesis occurred (Minguez-Mosquera and Gallardo-Guerrero 1996). The values on *Arabidopsis* indicates a rather complex situation. Thus, while at 35 and 44 DAS there was less activity in the younger leaf (with the exception of the oldest node), at 53 DAS high Chlase activity was in the younger leaves. This suggests that in non-senescent leaves activity is related to leaf age with higher activity in pre-senescent leaves than in younger ones but that senescence itself results in a reduction in activity. Hence, Chl *a* concentration is not directly correlated with Chlase activity. The same overall pattern was observed in the *eti 5* mutant, except that in this case senescence is delayed relative to WT. This is unsurprising since this mutant is insensitive to ethylene (Harpham *et al.* 1991) which can accelerate senescence in detached *Arabidopsis* leaves but not in the mutant. This insensitivity may regulate Chlase activity since other work (Trebitsh *et al.* 1993) indicates that ethylene can induce the synthesis of Chlase in oranges. Despite of recently finding, Chlase homologues from *Arabidopsis* (Tsuchiya *et al.* 1999), the relationship between Chlase and Chl content and the precise role of Chlase remain open.

References

Aljuburi, H., Huff, A., Hsheih, M.: Enzymes of chlorophyll catabolism in orange flavedo. - Plant Physiol. **63** (Suppl.): 410, 1979.

Arnon, D.: Copper enzymes in isolated chloroplast. Polyphenoloxidase in *Beta vulgaris*. - Plant Physiol. **24**: 1-15, 1949.

Bradford, M.: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Garcia, A.L., Galindo, L.: Chlorophyllase in *Citrus* leaves. Localization and partial purification of the enzyme. - Photosynthetica **25**: 105-111, 1991.

Harpham, N., Berry, A., Knee, E., Roveda-Hoyos, G., Raskin, I., Sanders, I., Smith, E., Wood, C., Hall, M.: The effect of ethylene on the growth and development of wild-type and mutant *Arabidopsis thaliana* (L.) Heynh. - Ann. Bot. **68**: 55-61, 1991.

Holden, M.: The breakdown of chlorophyll by chlorophyllase. - Biochem. J. **78**: 359-364, 1961.

Kuroki, M., Shioi, Y., Sasa, T.: Purification and properties of a soluble chlorophyllase from tea leaf sprouts. - Plant Cell Physiol. **22**: 717-725, 1981.

Matile, Ph., Schellenberg, M., Vicentini, F.: Localization of chlorophyllase in the chloroplast envelope. - Planta **201**: 96-99, 1997.

Minguez-Mosquera, M., Gallardo-Guerrero, L.: Role of chlorophyllase in chlorophyll metabolism in olives cv. Grodal. - Phytochemistry **41**: 691-697, 1996.

McFeeters, R., Chichester, C., Whitaker, J.: Purification and properties of chlorophyllase from *Ailanthus altissima* (tree of heaven). - Plant Physiol **47**: 609-618, 1971.

Ogura, N.: Studies on chlorophyllase of tea leaves II. Seasonal change of a soluble chlorophyllase. - Bot. Mag. (Tokyo) **82**: 392-396, 1969.

Scott, I.: Plant hormone response mutants. - Physiol. Plant. **7**: 147-152, 1990.

Šesták, Z.: Chlorophylls and carotenoids during leaf ontogeny. - In: Šesták, Z. (ed.): Photosynthesis During Leaf Development. Pp. 76-106. Academia, Praha; Dr. W. Junk Publ., Dordrecht - Boston - Lancaster 1985.

Shimizu, S., Tamaki, E.: Chlorophyllase of tobacco plants I. Preparation and properties of water soluble enzyme. - Bot. Mag. (Tokyo) **75**: 462-467, 1962.

Tanaka, K., Kakuno, T., Yamashita, J., Horio, T.: Purification and properties of chlorophyllase from greened rye seedlings. - J. Biochem. **92**: 1763-1773, 1982.

Trebitsh, T., Goldschmidt, E., Riov, J.: Ethylene induces *de novo* synthesis of chlorophyllase, a chlorophyll degrading enzyme, in *Citrus* fruit peel. - Proc. nat. Acad. Sci. USA **90**: 9441-9445, 1993.

Tsuchiya, T., Ohta, T., Okawa, K., Iwamatsu, A., Shimada, H., Masuda, T., Takamiya, K.: 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.