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BRIEF COMMUNICATION

Chlorophyllase activity and chlorophyll content in wild type and *eti 5* mutant of *Arabidopsis thaliana* subjected to low and high temperatures

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Abstract

Chlorophyll *a* (Chl *a*) content and chlorophyllase (Chlase) activity from leaves of wild type (WT) and the ethylene-insensitive mutant (*eti 5*) of *Arabidopsis thaliana* (L.) Heynh during temperature stress and plant recovery have been studied. The plants were subjected to temperatures of 4 °C (LT) and 38 °C (HT) for 24 h. Chl *a* gradually decreased somewhat during stress and in the first day of recovery, especially in HT-treated plants. At the end of the experimental period (1 d stress and 10 d recovery) Chl *a* content was lower in *eti 5* plants than in WT ones. The Chlase in WT was more affected than in *eti 5* plants during the temperature treatment and the recovery period.

Additional key words: chilling stress, ethylene-insensitive mutant, heat stress.

Chlorophyllase (Chlase) catalyzes the first step of chlorophyll (Chl) breakdown by hydrolysis of Chl into chlorophyllide and phytol (Holden 1961). Numerous reports indicate that Chlase has a role in Chl catabolism during leaf senescence and unfavorable environmental conditions (Garcia *et al.* 1980, Strother and Vatta 1986, Amir-Shapira *et al.* 1987, Majumdar *et al.* 1991).

Mutants of *Arabidopsis thaliana* are being used increasingly in physiological and biochemical studies (Scott 1990). Recently it has been found that wild type (WT) of *Arabidopsis thaliana* has higher Chlase activity than the ethylene-insensitive mutant (*eti 5*) (Todorov *et al.* 2003). The aim of the present study was to determine the changes of Chl and Chlase activity during temperature treatment and the recovery period in rosette leaves of WT and the *eti 5* mutant of *A. thaliana* and to assess the sensitivity of both types to low and high temperatures.

Seeds of WT and *eti 5* mutant of *Arabidopsis thaliana* (L.) Heynh were sown on a mixture of soil and *Perlite* 2:1 in plastic pots in a growth chamber. The growth conditions were: day/night temperature of 18/24 °C, irradiance of 90 μmol m^{-2} s^{-1}, and a 12-h photoperiod. Temperature stresses were carried out by subjecting plants to either 4 °C (LT) or 38 °C (HT) for 24 h, in darkness at the 40th day after sowing.

The sequence of procedures for determining the chlorophyllase (Chlase, EC 3.1.1.14) activity and Chl content have been described previously (Todorov *et al.* 2003). Briefly, the leaves were homogenized in 80 % acetone at 4 °C and centrifuged at 5 000 *g* for 10 min. The acetone extract was used for spectrophotomertric (*Spekol 11*, *Carl Zeiss*, Jena, Germany) determination of endogenous Chl (Arnon 1949) while the pellet was re-suspended in 5 mM potassium phosphate buffer (pH 7.0) containing 50 mM KCl, 0.24 % Triton X-100 and then centrifuged at 12 000 *g* for 10 min. The supernatant was used as a crude enzyme extract. The assay medium

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Abbreviations: Chl - chlorophyll; Chlase - chlorophyllase; *eti 5* - ethylene-insensitive mutant; HT - high temperature; LT - low temperature; WT - wild type.
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contained crude enzyme extract (250 mm^3), the above mentioned buffer (250 mm^3) and Chl *a* (*Sigma-Aldrich, Chemie GmbH*, Deisenhofen, Germany) (50 μg) dissolved in 100 % acetone (50 mm^3) and 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) and the mixture was shaken and then centrifuged at 8 000 *g* for 5 min to separate the phases. Chlorophyllide *a* was determined in the acetone phase spectrophotometrically at 667 nm (McFeeters *et al.* 1971). Chlase activity was expressed as production of chlorophyllide *a*. Proteins were determined by the method of Bradford (1976). The results presented are from two experiments, with three replicates of each determination. The results were analysed statistically using Fisher's criteria.

There was no significant effect of LT on Chl *a* content during the stress period (Fig. 1). On the other hand, HT caused its gradual reduction in both the plant types. During the recovery period, LT-treated WT plants showed an increase in Chl *a* and although there was an early reduction of Chl *a* in HT-treated WT plants, they had partly recovered by 240 h. Chlase activity showed little change in either WT or *eti 5* in the first 6 h of stress. However, there was an increase in Chlase activity in WT above the control levels after 12 h stress, especially in the HT-treated plants. After 24 h, Chlase activity in WT with both the stresses was much lower than in the control. In *eti 5* LT caused little change in Chlase activity during the stress but HT caused a significant fall 6 h after stress application followed by a marked increase after 12 h and a further decrease after 24 h. During the recovery period in WT both the LT- and HT-treated plants showed a marked increase in Chlase 72 and 144 h from the start of the experiment. LT-treated *eti 5* plants showed a similar trend except that the difference between *eti 5* control and treated plants was still large after 240 h of recovery. The HT-treated plants recovered to *eti 5* control levels by 48 h which were maintained until the end of the experiment.

Chlase activity varies with plant development and species (Šesták 1985). In young tea and tobacco it is considerably higher than in older leaves (Shimizu and Tamaki 1962, Ogura 1969, Kuroki *et al.* 1981) while in greening oranges Chlase and Chl content increase

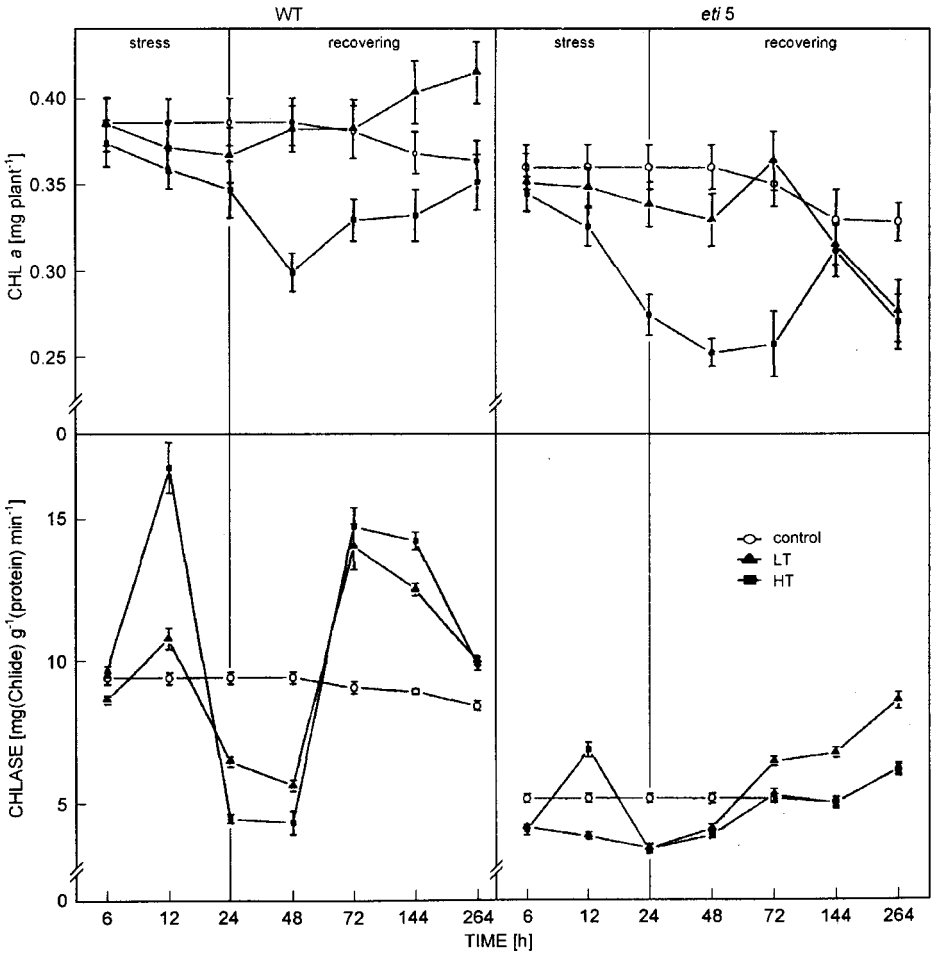

Fig. 1. Chl *a* content and Chlase activity of *A. thaliana* during temperature treatment and recovery periods. Lowest significant differences: 5 % = 0.014 (Chl *a*) or 0.711 (Chlase); 1 % = 0.018 (Chl *a*) or 0.962 (Chlase).

in tandem (Aljuburi *et al.* 1979). It has been found that the upper rosette leaves of *Arabidopsis* possess higher Chl content and lower Chlase activity, compared with lower ones (Todorov *et al.* 2003). The use of mutant plants and abiotic stress is an approach to understanding the relationship between enzyme activity and endogenous substrate. It has been found that Chlase activity rises during unfavourable environmental conditions causing Chl catabolism; thus, sublethal freezing of *Brassica napus* seeds leads to degreening because of the activation of Chlase (Johnson-Flanagan and McLachlan 1990b). It has also been shown that decrease in water content in the primary soybean leaves increases Chlase activity (Majumdar *et al.* 1991).

From our experiments with *A. thaliana* it can be concluded that Chl *a* content changes little in response to the stresses imposed although a significant effect of HT was observed. The results of the comparisons of Chlase activity with the Chl *a* content indicated that Chl content did not always correlate with enzyme activity. When Chlase increased, it caused Chl destruction 12 h after HT treatment while after 48-h recovery Chlase and Chl increased in tandem. At the end of the stress and during the first 24 h of the recovery period Chl *a* content of HT-treated plants was declining although there was lower Chlase activity in the treated plants. This suggests that perhaps another enzyme is involved in Chl catabolism such as chlorophyll peroxidase which has been proposed as an alternative system to Chlase (Huff 1982, Johnson-Flanagan and McLachlan 1990a). Alternatively, it may be that the activity monitored *in vitro* does not reflect *in vivo* activity.

In our experiments Chlase in the mutant responds less to stress than WT. This mutant is insensitive to ethylene which can accelerate senescence in detached *A. thaliana* WT leaves but not in *eti 5* (Harpham *et al.* 1991). Other work indicates that ethylene treatment can induce *de novo* synthesis of Chlase increasing its activity and leading to Chl destruction (Trebitsh *et al.* 1993). Besides, enhancement of ethylene production during senescence and unfavorable growth conditions has been established (Konze and Kwiatkowski 1981, Johnson-Flanagan and Spencer 1994). Lower Chlase activity of the mutant under both normal and stress conditions was probably due to its insensitivity to ethylene. In spite of recent findings concerning Chlase homologues from *Arabidopsis* (Tsuchiya *et al.* 1999), the relationship between Chlase and Chl content, and the precise role of Chlase remain open.

To summarise, Chlase activity in *A. thaliana* WT was affected much more severely by temperature treatment than in *eti 5,* although its Chl content was less changed. HT treatment provoked more significant changes in Chl content and Chlase activity than LT treatment.

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