学 位 論 文 内 容 の 要 旨

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学 位 論 文 題 名

Functional study on a common hydrolase enzyme in plants: Chlorophyllase contributes to a defense mechanism of plants against insect herbivores
（植物が共通してもつ加水分解酵素クロロフィラーゼの機能解析：クロロフィラーゼは草食昆虫に対する植物の防御応答に関与する）

Insect herbivores and plants have been living together for a long history. During co-evolution, plants have developed many different defense systems to protect themselves from insect herbivores. Nearly all classes of secondary metabolites were used as chemical defense compounds. However, whether chlorophyll (Chl)-derivatives can be used for defense against insect herbivores are unknown. Chlorophyllase (CLH), which catalyzes the release of the phytol chain from Chlorophyll (Chl) to produce chlorophyllide (Chlide), is long considered to be involved in the first step of Chl breakdown. However, Schenk et al presented evidence showing that CLH was not essential for Chl breakdown during dark-induced senescence [Schenk et al. FEBS Lett. 2007, 581: 5517-5525]. Arabidopsis contains two isoforms of CLH (CLH1 and CLH2), and it was hypothesized that under some stressful conditions or in the presence of methyl-jasmonate (MeJA) CLH1 is involved in Chl breakdown.

To examine the possible involvement of CLH1 in MeJA-induced Chl breakdown, we carefully analyzed that Chlide content in Arabidopsis leaves. We evaluated Chlide produced during Chl extraction by comparing different extraction methods with wild-type (WT) and mutant Arabidopsis leaves that lack the major isoform of CLH. The results suggested that almost no Chlide existed in leaves neither presence nor absence MeJA treatment. Therefore, CLH1 might not be involved in Chl degradation under MeJA treatment conditions.

It was suggested that during pigment extraction procedures with acetone, the phytol side chain of Chl is sometimes removed, forming Chlide, which affects Chl measurement when high performance liquid chromatography (HPLC) is employed for Chl analysis. Here, several extraction methods were compared to provide alternatives to researchers who utilize HPLC for the analysis of Chl levels. As a result, the following three methods are recommended. In the first method, leaves are briefly boiled prior to extraction. In the second method, grinding or homogenization of leaves is performed at sub-zero temperatures. In the third method, N, N'-dimethylformamide (DMF) is used for the extraction of pigments. When compared, the first two methods eliminated almost all Chlide-forming activity in *Arabidopsis thaliana* and other three tested species. However, DMF effectively suppressed the activity of CLH only in the leaves of Arabidopsis. All three methods evaluated in this study reduce the artifactual production of Chlide and are thus suitable for pigment extraction for HPLC analysis. The advantage and disadvantage of each method were discussed.

About the function of CLH, direct evidence was also collected. We analyzed the *clh* mutants which lack single or both CLH isoforms after MeJA-treatment. The results showed that the *clh* knockout lines were still able to degrade Chl at the same rate as wild types. Subsequently, by membrane fractionation and an analysis of the localization of the fusion of CLH1 and yellow fluorescent protein, we found that CLH1 was located outside the chloroplasts, and it was located to the tonoplast and endoplasmic reticulum (ER).

Later, we demonstrated that Chlide and pheophorbide (Pheide) were poisonous to the larvae of a common generalist *Spodoptera litura*. In addition, significant more larvae were dead and the development of the larvae was delayed by feeding the two Chl-derivatives. While by feeding the same concentration of Chl, survival ratio and development rate of the larvae could not show significant difference comparing with control. To mimic the leaf cells after they were eaten by larvae, leaves were homogenized both at PH8 and PH10. The Chlide amount

was subsequently analyzed by HPLC. The results showed that much Chlide was produced in the leaf homogenate of CLH1 overexpression lines and WT, while only little Chlide in *clh1-1* mutant. Finally we employed WT, *clh1-1* and 3 CLH1 overexpression lines to feed the larvae for 11 days. Although almost all of the larvae who ate WT and *clh1-1* were survived, the average survival rates of larvae were all significantly decreased after they ate the 3 CLH1 overexpression lines. Taken together, we conclude that CLH1 is not involved in Chl breakdown even during MeJA-induced senescence in Arabidopsis. Plants use Chl-derivatives for defense against insect herbivores through CLH activity. This system is quite convenient for defense against insect herbivores and widely exists in the plant kingdom.