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Functional study on a common hydrolase enzyme in plants: Chlorophyllase contributes to a defense mechanism of plants against insect herbivores

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LIST OF ABBREVIATIONS

Chl, Chlorophyll

Chlide, Chlorophyllide

CLH, Chlorophyllase

HPLC, High performance liquid chromatography

MeJA, Methyl-jasmonate

DMF, N, N'-dimethylformamide

YFP, Yellow fluorescent protein

Pheide, Pheophorbide

WT, Wild type

ER, Endoplasmic reticulum

PM, Plasma membrane

Suc, Sucrose

GENERAL ABSTRACT

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. 2007). 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 (Azoulay Shemer et al. 2008).

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 of 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 are 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).

Finally, we demonstrate here that Chlide and pheophorbide (Pheide) are poisonous to the larvae of a common generalist herbivore *Spodoptera litura*. In addition, significantly more larvae were dead and the development of the larvae was delayed by feeding Chlide and Pheide. While by feeding the same concentration of Chl, survival ratio and development rate of the larvae could not show significant difference comparing with the control. To mimic the leaf cells after they were eaten by larvae, the leaves were homogenized under either pH8 or pH10 conditions. The Chlide amount was subsequently analyzed by HPLC after the leaf homogenate was incubated at room temperature. The result showed that much Chlide was produced in the leaves mixture of CLH1 overexpression lines and WT, while only little Chlide in the *chl1-1* mutant. Finally we fed *Spodoptera litura* with WT, *chl1-1* and three CLH1-overexpression lines for 11 days. Although almost all of the larvae who ate WT and the *chl1-1* mutant were survived, the average survival rates of larvae were significantly decreased after they ate the leaves of three CLH1-overexpression lines. Taken together, we conclude that CLH1 is not involved in Chl breakdown even during MeJA-induced senescence in Arabidopsis. However, we demonstrate that 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.

Keywords: Chlorophyllase; Methyl jasmonate; HPLC; Chlorophyll-derivatives;
Insect herbivore; Defense

CHAPTER 1: GENERAL INTRODUCTION

1.1 Chlorophyll degradation and chlorophyllase

In our common home, the earth, the color change is one of the most significant events. The appearance of chlorophyll (Chl) in terrestrial plants during springtime and its disappearance in autumn that can even be seen from outer space (Eckhardt et al. 2004). Therefore, Chl biosynthesis and degradation would be very important biochemical reactions for land plants and algae. Although the research of Chl degradation started much later than Chl biosynthesis research, almost all enzymes which were proposed to involve in Chl breakdown have been identified (Tsuchiya et al. 1999) (Pružinská et al. 2003) (Pružinská et al. 2007) (Schelbert et al. 2009), except for Mg-dechelataase which catalyze the conversion of Chl *a* to pheophytin *a* by removing Mg^{2+} . Among them the function of chlorophyllase (CLH) in Chl degradation is controversial. CLH is the enzyme which catalyze the conversion of Chl to chlorophyllide (Chlide) by removing the phytol side chain. Since CLH was discovered in 1912 (Stoll 1912), for a long time, CLH was believed to be the first step of Chl breakdown (Hendry et al. 1987) (Drazkiewicz and Krupa 1991) (A Tanaka and R Tanaka, 2006) (Hörtensteiner, 2006), because the activity is very high and is conserved in almost all of the higher plants and green algae (Barrett and Jeffrey 1964) (Drazkiewicz and Krupa 1991) (MCM Chen et al. 2012). However, when the Arabidopsis CLH knockout mutants are available, Schenk and coworkers found CLH was not essential for dark induced-senescence-related Chl

breakdown (Schenk et al. 2007). However, another research group presented evidence to indicate that citrus CLH was responsible for ethylene-induced fruit color break (Azoulay Shemer et al., 2008). In *Arabidopsis*, there are two isoforms of CLH which are named as chlorophyllase1 (CLH1) and chlorophyllase2 (CHL2) (Tsuchiya et al. 1999). Although the expression of CLH2 is constitutive, The CLH1 expression level is sharply induced by MeJA, wounding and coronatine (a toxin produced by the bacterium *Pseudomonas syringae*) (Benedetti et al. 1998) (Tsuchiya et al. 1999). Therefore, it was suggested that CLH1 involved in Chl degradation under stress or phytohormone-induced leaf senescence (Azoulay Shemer et al. 2008).

On the other hand, the reported levels of Chlide was considerably varied even in the same *Arabidopsis* wild-type (WT) line (columbia) among literature (Benedetti and Arruda 2002) (Kariola et al. 2005) (Yang et al. 2012) (Schenk et al. 2007). If the levels of Chlide fluctuates in vivo under different conditions, it would be reasonable to assume that CLH is involved in chlorophyll degradation under some conditions, under which CLH expression level is enhanced.

1.2 Plant defense against insect herbivores

Plants and insect herbivores have been living together for more than 350 million years (War et al. 2012). Although plants cannot move freely like animals, they are

never just passively eaten by insect herbivores. In fact, plants are using many strategies to defense against insect herbivores. Some plants have developed structural defense systems, such as trichomes, thick leaves with wax, spinescent leaves or stems. These structures can discourage consumption (Hanley et al. 2007). On the other hand, almost all of the plants have evolved a plethora of different chemical defenses. The compounds of chemical defenses with chemical properties that directly deter herbivores from feeding on a plant. Although some heavy metal can be concentrated from the environment by some plant species for defense, such as lead, zinc and nickel (R Boyd 2012). In most cases, chemical defenses are known as organic chemical defenses. Organic chemical compounds which are produced by plants as secondary metabolites are often used for defense against insect herbivores. They cover nearly all classes of (secondary) metabolites: some are constitutive; others are inducible by insect attack (Wittstock and Gershenzon 2002) (Mithöfer and Boland 2012). Some compounds act indirectly via the attraction of the predators of insect herbivores, also known as volatile organic chemicals (Dicke et al. 1990) (A Kessler and IT Baldwin 2001) (Degenhardt 2009). While many others act directly on the insect herbivores (Mithöfer and Boland 2012). In this group, many secondary metabolites which are well known as defense compounds including Alkaloids, Cyanogenic glycosides, terpenoids, phenolics and so on. In addition, amino acids and even peptides are also used as a

defense (T Huang et al. 2011) (Habib and Fazili 2007). As Chl degradation intermediate, Chl-derivatives' other physiological function was largely unknown. It was reported that Pheide *a* possess cytotoxic activity to cancer cell (Sowemimo et al. 2012). In Arabidopsis, light-independent cell death can be induced by accumulation of Pheide *a* (Hirashima et al. 2009). However, whether plants use Chl-derivatives for defense against insect herbivores have not reported yet.

1.3 The aims and main finding of the investigation

Accurate analysis the content of Chl and its derivatives by high performance liquid chromatography (HPLC) is one of the basic technology in the Chl metabolite research field nowadays. However, during Chl extraction and HPLC analysis, artifactual productions are easily formed by enzymatic or non-enzymatic reaction other than the true content in the leaves. In chapter 2, I noticed that artifactual Chlide production is easily formed during Chl extraction by normal extraction methods. I evaluated the artifactual Chlide production during Chl extraction by comparing different extraction methods with wild-type and mutant Arabidopsis leaves that lack the major isoform of CLH. Finally, several methods were compared to provide alternatives to researchers who utilize HPLC for the analysis of Chl levels without artifactual Chlide production. Three other species *Glebionis*

coronaria, *Pisum sativum* L. and *Prunus sargentii* Rehd. were also used to test these methods.

In chapter 3, I clarified the function of CLH. Firstly, I demonstrated that CLH1 was not involved in Chl breakdown with methyl-jasmonate (MeJA) treatment, even when the expression level of CLH1 was much induced. On the other hand, both CLH1-YFP (C-terminal fusion) and the native CLH1 was located outside of the chloroplast. I subsequently found plants utilize Chl-derivatives (Chlide and Pheide) for defense against insect herbivores through CLH activity, only after plant leaves were eaten by insect herbivores. This is the first report demonstrated that plants use Chl-derivatives for defense against insect herbivores.

**CHAPTER 2: SIMPLE EXTRACTION
METHODS THAT PREVENT THE
ARTIFACTUAL CONVERSION OF
CHLOROPHYLL TO CHLOROPHYLLIDE
DURING PIGMENT ISOLATION FROM LEAF
SAMPLES**

ABSTRACT

High performance liquid chromatography (HPLC) is becoming widely used for the measurement of photosynthetic pigments because it provides an accurate determination of a variety of photosynthetic pigments simultaneously. This technique has a drawback compared with conventional spectroscopic techniques, because it is more prone to structural modification of pigments during extraction, thus potentially generating erroneous results. During pigment extraction procedures with acetone, the phytol side chain of chlorophyll (Chl) is sometimes removed, forming chlorophyllide (Chlide), which affects chlorophyll measurement using HPLC. We evaluated the artifactual Chlide production during Chl extraction by comparing different extraction methods with wild-type (WT) and mutant *Arabidopsis* leaves that lack the major isoform of CLH. 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 and homogenization of leaves are 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*, *Glebionis coronaria*, *Pisum sativum* L. and *Prunus sargentii* Rehd. However, DMF effectively

suppressed the activity of CLH only in the leaves of *Arabidopsis*. Chlide production in leaf extracts is predominantly an artifact. All three methods evaluated in this study reduce the artifactual production of Chlide and are thus suitable for pigment extraction for HPLC analysis. The boiling method would be a practical choice when leaves are not too thick. However, it may convert a small fraction of Chl *a* into pheophytin *a*. Extraction at sub-zero temperatures is suitable for all plant species examined in this study but may be tedious for a large number of samples and it needs liquid nitrogen and equipment for grinding leaves. Using DMF as an extractant is simple and suitable with *Arabidopsis* samples. However, this solvent cannot completely block the formation of Chlide in thicker leaves.

Key words: chlorophyll extraction, chlorophyllide, chlorophyllase

2.1 Introduction

Chlorophyll (Chl) analysis has been conducted in numerous studies due to the importance of this pigment in the physiology of plants. Chl is involved in the absorption and transfer of light energy, and electron transfer, all of which are vital processes in photosynthesis. Chl content can change in response to biotic and abiotic stresses such as pathogen infection (Mur et al. 2010), and light stress (Brouwer et al. 2012), (Kitajima and Hogan 2003). Thus, quantification of Chl provides important information about the effects of environments on plant growth (Bianchi and Findlay 1990) (Rosevear et al. 2001) (Oserkowsky 1933) (Cartelat et al. 2005) (Schlemmer et al. 2005).

Historically, spectroscopic methods have been most frequently used for Chl measurement because they provide a quick, accurate and inexpensive estimation of Chl concentration (Arnon 1949) (Lichtenthaler 1987) (Porra et al. 1989). However, conventional spectroscopic methods, where bulk photosynthetic pigments are measured in the same cuvette, have limitations in their ability to simultaneously measure multiple photosynthetic pigments due to the overlapping absorption spectra of these pigments. For this reason, it has become more common to separate photosynthetic pigments by high-pressure liquid chromatography (HPLC) prior to spectrophotometric analysis (Z Li et al. 2009) (EH Kim et al. 2009). When separating pigments by HPLC, extra care must be taken since HPLC analysis is

prone to the artifactual modification of pigments. In particular, cleavage of the phytol chain of Chl molecules readily occurs with the use of common extraction solvents such as 80% acetone (Venketeswaran 1965). The products of Chl hydrolysis are chlorophyllide (Chlide) and free phytol. Since Chlide has the same absorption spectra as Chl in the visible light spectrum and phytol does not, cleavage of the phytol chain does not affect the values obtained using conventional spectroscopic methods of Chl determination when samples are extracted with organic solvents. However, due to the polar nature of Chlide, it is readily separated from Chl with HPLC, and thus the artifactual formation of Chlide can result in erroneous data using HPLC-based determination of Chl concentration.

Conversion of Chl to Chlide induced by the extraction agent reduces the apparent concentration of Chl in samples. It is usually difficult to distinguish whether or not the Chlide detected during HPLC analysis is an artifact or a natural product. In fact, Chlide has been considered a natural product in leaves without examining the basis of its formation (Benedetti and Arruda 2002), (Kariola et al. 2005). In order to avoid possible misinterpretation of Chl levels, it is essential to employ extraction methods that result in a minimal amount of conversion of Chl to Chlide.

It has been reported that the hydrolase enzyme, chlorophyllase (CLH) catalyzes the formation of Chlide during pigment extraction (Bacon and Holden 1967) (Fig. 2-

1A). This enzyme is unusually stable in high concentrations of organic solvents such as 50-70% aqueous acetone (Holden 1961) (Mcfeeters et al. 1971). Higher plants contain one or two isoforms of this enzyme (Hörtensteiner, 2006) and Arabidopsis has two CLH isoforms encoded by *CLH1* and *CLH2* genes, respectively (Tsuchiya et al. 1999). *CLH1* encodes the isoform of CLH that accounts for the majority of CLH in Arabidopsis leaves. *CLH1* gene expression is significantly upregulated by methyl-jasmonate (MeJA), a phytohormone mediating various biotic and abiotic signaling pathways (Cheong and Choi 2003). In contrast, *CLH2* is constitutively expressed and only represents a minor fraction of CLH activity (Schenk et al. 2007). In the present study, we assessed how much Chlide is formed during pigment extraction compared to the amount that naturally occurs in leaves. In a subsequent analysis, we then examined whether or not CLH is involved in Chlide formation during extraction by comparing its formation in leaves of wild-type and an Arabidopsis mutant which is deficient in CLH activity. Collectively, these experiments indicated that the majority of Chlide detected in extracts obtained using 80% acetone or pure acetone is produced during pigment extraction through the reaction catalyzed by CLH. We also compared three different methods of pigment extraction that were previously reported in literature. Bacon and Holden (Bacon and Holden 1967) reported that CLH activity could be suppressed by boiling leaves for a period of 5 min. They also indicated, however,

that the boiling treatment also removes Mg^{2+} from Chl (Bacon and Holden 1967). We found that, in the case of Arabidopsis leaves, CLH can be inactivated and Mg^{2+} removal from Chl can be reduced when samples were only boiled for 5 sec. In the method of Schenk et al (Schenk et al. 2007), leaves were first ground into powder in liquid nitrogen and pigments were subsequently extracted in buffered acetone cooled to $-20^{\circ}C$. We found this method is very efficient when processing a relatively small number of samples. Finally, we tested the use of N, N'-dimethylformamide (DMF) as an extraction agent to eliminate the formation of Chlide during sample preparation. Although Moran and Porath (Moran and Porath 1980) reported that Chl is stable in this solvent, they did not characterize the effect of DMF on Chlide formation. In our study, DMF was capable of extracting pigments without enabling the conversion of Chl to Chlide in Arabidopsis, however, for the other species which we have tested in this study, DMF cannot completely suppress the activity of CLH. Collectively, all three methods (boiling leaf sample, freezing leaf samples in liquid nitrogen with the use of pre-cooled acetone, and the use of DMF as an extraction agent) were superior to the methods only using 80% or pure acetone for the extraction of photosynthetic pigments. It is important to understand pros and cons of each method and choose an appropriate one for each plant species and for the purpose of pigment analysis.

2.2 Methods

2.2.1 Chemicals

Acetone (HPLC grade, 99.7% purity) was purchased from Wako Pure Chemical Industries, Ltd, Osaka, Japan. DMF (Guaranteed Reagent Grade), ethanol (HPLC grade) and other solvents (Guaranteed Reagent Grade) were purchased from Nacalai Tesque, Inc., Kyoto, Japan.

2.2.2 Plant materials

WT *Arabidopsis* (Columbia-0 ecotype) and a T-DNA insertion line (SALK_124978; designated as *chl1-1*, (Schenk et al. 2007)) was primarily used in this study. Plants were grown in soil under long-day conditions (16 h light/8 h dark) in growth chambers under fluorescent light ($70\text{--}90\ \mu\text{mol photons m}^{-2}\text{s}^{-1}$) at 23°C. For pigment extraction, leaves (7th to 9th leaves counting from the bottom of the plant) were harvested either after 4 weeks or after a period of 8 weeks to allow natural senescence. For the MeJA treatment, 4-week-old plants were sprayed with 100 μM MeJA in 0.1% ethanol, 0.01% Tween 20, or solvent control (0.1% ethanol, 0.01% Tween 20), and kept for 3 days at the same growth conditions. For dark-induced senescence, the 7th-9th leaves of 4-week-old plants were detached and placed on wet filter paper (3 mM MES buffer, pH 5.8, with or without 50 μM MeJA) and incubated in complete darkness at 23°C for up to 3 days. In addition to *Arabidopsis*, three other plant species were tested in this study. *Glebionis*

coronaria (garland chrysanthemum) adult plants were purchased from a supermarket, and their mature leaves were used for the experiments. *Pisum sativum* L. (pea) was grown in soil under long-day conditions (16 h light/8 h dark) for seven days in growth chambers under fluorescent light (70-90 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) at 23°C. Then, young leaves were harvested for pigment extraction. Young leaves of *Prunus sargentii* Rehd. (North Japanese hill cherry) were collected from the campus of Hokkaido University, Japan in mid-May.

2.2.3 Chlorophyll extraction and analysis

Leaves were harvested and the fresh weight (18-30 mg) of each sample was recorded. Leaves were then frozen in liquid nitrogen and stored at -80°C in a deep freezer. In most experiments described in this study, pigments were extracted by immersing leaves in organic solvents for 10 to 48 hours. Incubation time, and the organic solvent were varied experiment to experiment, which is described in the result section. The procedure described below is common to all extraction methods used in this study unless otherwise noted. Firstly, tubes with leaves were removed from the liquid nitrogen and 1 ml of organic solvents cooled to 4°C or -30°C was immediately added to each tube and incubated at 4°C in the dark for 12 h for *Arabidopsis*, 20 h for *G. coronaria*, 48 h for pea and 10 h for cherry leaves. The length of incubation was determined for each plant species by preliminary experiments. For *Arabidopsis* leaves, the results of longer incubation in acetone at

-30°C in the dark for 4 days were described in the results section as well. The 80% acetone employed in this study contained 20% (v/v) 0.2 M Tris-HCl pH 8.

In the boiling method, the leaves were dipped into boiling water for 5 or 10 sec. The leaves were then placed on filter paper to absorb excess water and then homogenized in pure acetone at room temperature. Alternatively, four-degree acetone was added to the boiled samples in a 2-ml microtube. Tubes were then kept at 4°C for 12 h for *Arabidopsis*, 20 h for *G. coronaria*, 48 h for pea and 10 h for cherry leaves. After incubation, extracts were transferred to a glass vial and analyzed using HPLC as described below.

In the second extraction method, acetone was cooled to -30°C prior to its use. An aluminum metal box (BioMedical Science Co. Ltd., Tokyo, Japan) for holding sample tubes during shaking was cooled in liquid nitrogen for 30 min prior to its use. Two-ml microcentrifuge tubes containing leaf samples and homogenization beads were frozen in liquid nitrogen and then stored at -80°C. Acetone was added to the tubes containing the frozen samples while they were in the nitrogen-cooled metal box. Homogenization of the tissue was performed immediately by shaking the sample tubes containing the homogenization beads and leaf samples in an automatic bead shaker (Shake Master, BioMedical Science Co. Ltd, Tokyo, Japan).

In the third extraction method, 1 ml of DMF cooled to 4°C was immediately added to frozen leaves. The samples were then incubated at 4°C in the dark for 12 h for *Arabidopsis*, 20 h for *G. coronaria*, 48 h for pea and 10 h for cherry leaves. Subsequently, the organic solvent was recovered by centrifugation and its pigment composition was determined by HPLC as described below.

2.2.4 HPLC separation of photosynthetic pigments

The microtubes containing the homogenized samples were subjected to centrifugation at 15,000 rpm for 5 min at 4°C and the resulting supernatant was analyzed by HPLC with a Symmetry C8 column (150 mm in length, 4.6 mm in i.d.; Waters, Milford, MA, USA) according to the method of Zapata et al. (Zapata et al. 2000) . Elution profiles were monitored by measuring absorbance at 410 nm. Pigments used as standards were purchased from Juntec Co. Ltd. (Odawara, Japan).

2.2.5 Time course experiments

Leaf samples from 4-week-old *Arabidopsis* plants were immersed in pure acetone in 2.0 mL-microtubes that were held in a metal box that had been pre-cooled with liquid nitrogen. The sample tubes were then transferred to a tube rack and incubated at ambient temperature for the times indicated in Figure 4. After a prescribed time, the tubes were returned to the nitrogen-cooled metal box to terminate the incubation. Pigments were extracted from samples at a sub-zero

temperature while tubes were in the cooled metal box by adding stainless beads and shaking the box in a bead shaker (Shake Master).

2.3 Results

2.3.1 Use of 80% and pure acetone as extraction solvents results in the formation of Chlide

It was our objective to provide a simple and reliable method to extract chlorophyll for HPLC analysis that would be free from artifacts. In order to achieve this goal, we started with one of the simplest methods to extract pigments and attempted to improve it. We first compared the two conventional methods in which pigments are extracted from *Arabidopsis* leaf samples by soaking them in 80% or pure acetone for 12 hours at 4°C. Since CLH has been reported to be active in aqueous acetone but precipitated in pure acetone (Holden 1961) (Mcfeeters et al. 1971), it was expected that Chlide would only be produced in the 80%-acetone extracts. In line with this expectation, we determined that nearly 70% of the combined Chl and Chlide *a* content was composed of Chlide *a* in the 80%-acetone extracts (Fig. 2-2). In contrast, only a small amount of Chlide *a* was produced in pure acetone (Fig. 2-2). Therefore, it is likely that most of the Chlide *a* detected in 80% acetone was formed during extraction or after extraction. Since Chl *a* is much more abundant than Chl *b* and the trend of Chlide *b* formation was similar to that of Chlide *a*, we only describe the results on Chl *a* and Chlide *a* in the present study.

CLH activity increases in leaves that are either senescent (MT Rodríguez et al. 1987) or wounded (Kariola et al. 2005), and in response to MeJA treatment (Tsuchiya et al. 1999). Thus, we examined Chlide formation using naturally-senescent leaves from 8-week-old *Arabidopsis* plants, those in which senescence was induced by a 4-day dark treatment, and those in which Chl breakdown was induced by MeJA. Eight to ten percent of the Chl content in naturally-senescent, dark-treated, and MeJA-treated leaves was composed of Chlide (Figure 2-3) even when pure acetone was used. These results indicate that pure acetone does not sufficiently suppress the formation of Chlide during Chl extraction.

2.3.2 CLH is responsible for the formation of Chlide during Chl extraction

The experiments described above indicated that the majority of Chlide is formed during extraction. In the next series of experiments, we examined the time-course of Chlide formation during acetone extraction. We also tested whether or not CLH was involved in Chlide formation in pure acetone by comparing Chlide formation during extraction using *Arabidopsis* leaves from WT and a *clh1-1* mutant that lacks the major isoform of CLH. After immersing leaves in pure acetone, the leaves were incubated in pure acetone for up to 6 min (Fig. 2-4). In this series of experiments, Chlide formation was also compared in leaves from WT plants that had been either treated or not with 50 μ M MeJA because the CLH activity is more evident after MeJA treatment (Fig. 2-4).

Production of Chlide was negligible unless incubation was at an ambient temperature (Fig. 2-4). Increasing incubation time at an ambient temperature resulted in elevated amounts of Chlide (Fig. 2-4). MeJA-treated WT leaves yielded a higher amount of Chlide (up to 12% of the total Chl plus Chlide after 6 min of incubation) compared to non-treated WT leaves. In contrast, extracts from leaves from *chl1-1* plants only contained a trace amount of Chlide with or without MeJA treatment (Fig. 2-4). These results are consistent with the report of Schenk et al. (Schenk et al. 2007), in which they detected a much lower level of Chlide in acetone extracts of dark-incubated *chl1-1* leaves compared to extracts from dark-incubated WT leaves. These results indicate that the major isoform of CLH is responsible for Chlide formation during extraction.

2.3.3 Stability of Chl and Chlide in pure acetone

In our next line of investigation, we tested the stability of Chl and Chlide in pure acetone. Chlide production was evident from an extraction of WT leaves in pure acetone at ambient temperatures for 6 min (Fig. 2-4 and Table 2-1). Specifically, the pigments were extracted by grinding the leaves in pure acetone with stainless steel balls and the extracts were separated from cell debris by centrifugation. This procedure yielded approximately 200 nmol/gFW (fresh weight) Chlide in the extract. In contrast, Chlide levels were less than 10 nmol/gFW in the absence of the 2 – 6 min incubation. Extracts were also maintained for one day in darkness at

room temperature and their Chl and Chlide levels were measured using HPLC. No significant changes in Chl or Chlide levels were observed in the one-day-old extracts (Table 2-1). Therefore, it is likely that the majority of Chlide detected in the extracts were formed during the extraction or homogenization procedures.

2.3.4 Chlide formation is suppressed with rapid boiling of leaves

In order to provide a simple Chl extraction method that is less affected by CLH activity, we assessed if CLH activity could be inactivated by boiling leaves. Bacon and Holden (Bacon and Holden 1967) have already reported that CLH activity can be suppressed by boiling leaves for 5 min. However, they also found that this treatment destroys some pigments. Therefore, we examined whether shorter (approximately 5 or 10 sec) periods of boiling can adequately suppress CLH activity while avoiding pigment decomposition. In this experiment, mature *Arabidopsis* leaves were sprayed with 50 μ M MeJA and subsequently harvested. After collection they were dipped in boiling water for 5 or 10 sec, and then soaked in pure acetone. For pigment extraction, leaves were homogenized in acetone using stainless beads or kept in pure acetone overnight at 4°C.

Leaves from WT plants that were homogenized without boiling yielded 94 nmol/gFW and 46 nmol/gFW Chlide *a* when they were treated or not with MeJA, respectively, prior to extraction (Fig. 2-5A). In contrast, Chlide *a* was below a

detectable level in leaves that were not treated with MeJA when the leaves were boiled for 5 or 10 sec before Chl extraction. When MeJA-treated WT leaves were boiled for 5 or 10 sec prior to extraction, only 3.6 and 1.4 nmol/gFW Chlide *a* were detected, respectively (Fig. 2-5A). The combined sum of Chl and Chlide were not affected by boiling (Fig. 2-5A and 2-5B), indicating that the significant pigment losses, observed when leaves were subjected to 5 min boiling (Bacon and Holden 1967) , did not occur when the brief boiling procedure was used. The overall profiles of detectable photosynthetic pigments obtained by HPLC were not altered by boiling except for pheophytin *a*. This pigment increased slightly from 30 nmol/gFW to 50 nmol/gFW in boiled samples. These levels represented approximately 0.2% to 0.3% of total Chl *a* levels, and are almost negligible in the HPLC profiles (Fig. 2-6B).

To further simplify the extraction method, boiled leaves were kept overnight in pure acetone at 4°C. Chlide formation in both MeJA-treated and non-treated boiled WT leaves were negligible after overnight incubation (Fig. 2-5C and 2-5D), indicating that rapid boiling almost completely inactivated CLH activity.

2.3.5 Pure acetone extraction at sub-zero temperatures

Schenk et al. (Schenk et al. 2007) have already demonstrated that Chlide formation could be minimized if leaf samples were ground in liquid nitrogen and extracted

with acetone cooled to -20°C. In this study, we attempted to simplify their method. We evaluated whether or not Chl could be extracted just by immersing frozen leaves in pure acetone cooled to -30°C. Our data indicated that a substantial amount of Chl remained in leaf tissue after an overnight incubation in -30°C acetone (data not shown). After 4 days of incubation, the majority of Chl had been extracted as evidenced by the white appearance of extracted leaves (data not shown). HPLC analysis showed that only trace amounts of Chlide were detected in samples obtained from WT leaves that had been treated or not with MeJA (Fig. 2-6 and 2-7), indicating that CLH activity was negligible at -30°C.

2.3.6 Extraction of Chl by DMF or ethanol

DMF is reported to efficiently extract Chl without the need for homogenization (Inskeep and Bloom 1985). In the present study, we tested whether or not Chlide formation occurs during extraction with DMF. WT leaves, treated or not with MeJA, were incubated for 12 h in DMF at 4°C. Only a trace amount of Chlide was detected (Fig. 2-7). Modification of pigment structure that impacts the profiles obtained by HPLC separation of major photosynthetic pigments, including Chl *a*, did not occur with the use of DMF (Fig. 2-6).

We also examined the ability of ethanol to extract chlorophyll in this study. WT leaves, those were either treated or not with MeJA, were incubated for 12 h in ethanol at 4°C. This solvent did not extract all of the chlorophyll from leaves after

a 12-h incubation, as evidence by the leaves retaining some greenish color. Additionally, this solvent induced significant modifications of the pigments (Fig. 2-6).

2.3.7 Comparison of the Chl extraction methods in different plant species

For testing the general utility of the three methods described as above, Chl was extracted from the leaves of three other plant species, namely, *Glebionis coronaria* (garland chrysanthemum), *Pisum sativum* L. (pea) and *Prunus sargentii* Rehd. (North Japanese hill cherry) (Fig. 2-8A-F). Chlide *a* was detected in all three species when pigments were extracted by homogenizing leaves in pure acetone at room temperature or by immersing leaves in 4°C pure acetone. These results indicate that all three species possess CLH activity. Among these species, the largest accumulation of Chlide was observed with pea leaves when the pigments were extracted by immersing leaves in pure acetone at 4°C, which converted 20% of Chl *a* to Chlide *a* (Fig. 2-8D). In contrast, the *G. coronaria* leaves did not show high CLH activity, which yielded only 2% of Chlide *a* compared to total Chl *a* levels by the acetone immersing method (Fig. 2-8B). The sub-zero temperature extraction yielded negligible amounts of Chlide from all three samples (Fig. 2-8A-F), demonstrating that the Chlide formed during the other extraction methods was predominantly an artifact. The boiling method worked well with the leaves of *G. coronaria*, which formed negligible amounts of Chlide *a* (Fig. 2-8A and 2-8B).

This method resulted in the formation of small amounts Chlide *a* with pea and cherry leaves (Fig. 2-8C-F). The Chlide *a* levels in this method were in a similar range with *Arabidopsis*, which was about 1% or less of total Chl *a* (Fig. 2-8D and 2-8 F). The DMF extraction method did not work as well for *G. coronaria*, pea and cherry leaves as it did for *Arabidopsis*. This method allowed formation of Chlide *a* up to 10% of total Chl *a* in pea leaves (Fig. 2-8D). Moreover, immersing leaves in DMF (48h) and acetone extracted only half of pigments compared to other methods (Fig. 2-8C). Interestingly, short boiling before immersing leaves in pure acetone drastically improved the extraction efficiency of pigments (Fig. 2-8C).

2.4 Discussion

It has been reported that the commonly used method of extraction of photosynthetic pigments with aqueous acetone sometimes results in artifactual Chlide formation (Holden 1961) (Schenk et al. 2007). In the present study, we determined the quantity of Chlide formation before, during or after extraction of these pigments using several different methods of extraction. By suppressing CLH activity during extraction, we demonstrated that only trace amounts of Chlide, if any, are present in the cells prior to extraction (see Fig. 2-4, 2-5, 2-7 and 2-8). We also showed that both Chl and Chlide are stable in acetone after extraction (Table

2-1). Therefore, it is unlikely that Chlide is formed in the solvents after the extraction procedure is completed. Based on our collective results, we concluded that Chlide is formed during the extraction process. We speculate that Chlide is formed when acetone infiltrates the tissue, or when the tissue is homogenized in acetone. During these processes, the actual concentration of acetone to which cells are exposed may increase gradually rather than immediately, thus allowing an opportunity for aberrant enzymatic reactions to occur. Although CLH is known to precipitate in pure acetone, it is capable of remaining highly active in lower concentrations of aqueous acetone (Tsuchiya et al. 1997) (Holden 1961). Therefore, it is likely that CLH catalyzes the formation of Chlide during extraction until the actual acetone concentration reaches nearly 100%. This hypothesis explains the differential effects of DMF on Chlide formation during Chl extraction from different plant species (Fig. 2-7 and 2-8). DMF suppressed Chlide formation in *Arabidopsis* leaves almost perfectly, while it allowed Chlide formation in other plant species whose leaves are thicker than *Arabidopsis* (Fig. 2-8). These observations can be explained by assuming that DMF infiltrate slower in thicker leaves than in thinner leaves.

The hypothesis mentioned as above raises the question why CLH is active only after the tissue is homogenized with organic solvents or soaked in organic solvents. A possible answer to this question may be that CLH is active in cells but separated

from Chl in cells. Schenk et al. (Schenk et al. 2007) used CLH-GFP targeting experiments and confirmed that CLH is localized outside of chloroplasts. If CLH is indeed separated from Chl in intact cells, the homogenization of cells or immersion of tissue in acetone may disrupt cell structures and enable CLH to act on Chl.

Chlide has been long considered to be an intermediate of both Chl biosynthesis and breakdown (KI Takamiya et al. 2000) ([Hörtensteiner, 2006](#)). Hörtensteiner and co-workers (Schenk et al. 2007) (Schelbert et al. 2009), however, suggested that Chlide is not a true intermediate of Chl breakdown, at least during leaf senescence in Arabidopsis. Instead, they indicated that Chl is degraded via pheophytin (Fig. 2-1B). Our results are consistent with the Chl breakdown model of Hörtensteiner and coworkers (Schenk et al. 2007) (Schelbert et al. 2009). The majority of Chlide detected in acetone extracts of leaf pigments in our experiments were formed by the action of CLH during extraction (see Fig. 2-3 and 2-4). These results suggest that plants only accumulate a small amount (if any) of Chlide in cells under either normal growth conditions ((Schenk et al. 2007) and this study) or when exposed to MeJA.

We compared three methods that suppress CLH activity with a conventional acetone-extraction method. In the first method, Arabidopsis leaves were boiled for

a short time (5 or 10 sec). This procedure almost completely suppressed Chlide formation with *Arabidopsis* and *G. coronaria* leaves. Bacon and Holden (Bacon and Holden 1967) already reported that a 5 minute period of boiling eliminates Chlide formation. Their boiling time, however, appears to have been too long since they observed extensive decomposition of the pigments (Bacon and Holden 1967). In principle, the boiling time used in this procedure should be optimized for each plant species but we do not suggest boiling leaves for more than 10 sec for most plant species (see Fig. 2-8). Thicker leaves may necessitate a longer boiling time. For example, we found that a 30 sec boiling time worked well to eliminate CLH activity in mulberry leaves in our laboratory (data not shown). This method appears to have another advantage in increasing the extraction efficiency of pigments from thicker leaves such as pea leaves when pigments are extracted by immersing leaves in organic solvents (see Fig. 2-8C). Thus, the boiling method combined with the use of DMF as an extractant would be worth testing when pigments are extracted from thicker leaves. A possible drawback of the boiling method is the potential for additional types of modification to Chl molecules. For instance, we observed a slight increase in pheophytin *a* concentration in our extracts (Fig. 2-6) indicating that 0.1 to 0.2% Chl *a* might be converted to pheophytin *a* by boiling. Thus, the boiling method is recommended in studies where the quantitation of pheophytin *a* is not being considered.

In the second method, frozen leaves were ground at sub-zero temperatures in a metal box that was cooled with liquid nitrogen. The leaves were then homogenized in pure acetone cooled to -30°C using an automatic bead shaker, Shake Master. The use of this shaker facilitates the processing of a relatively large number of samples. It is also possible to use cooled mortar and pestles for grinding leaves at sub-zero temperatures. However, this approach may be laborious and time-consuming when the analysis of a large number of samples is required. In addition, the recovery of a sufficient amount of solvent from a mortar can be problematic when only a small amount of sample tissue is used or available (Moran and Porath 1980). Therefore, the usage of a mortar and pestle with this method is recommended only when a relatively small number of samples need to be analyzed and when a sufficient amount of tissue is available for each sample. Another limitation of this method will be a requirement of liquid nitrogen, which might not be readily available in field research. Regardless of these limitations, this method is superior to other methods in completely suppressing CLH activity in all plant species tested in this study. This method would be suitable for determining the minimum levels of Chlide formation.

In the third method, pigments were extracted with DMF. This solvent has been previously used for pigment extraction (Moran and Porath 1980), (Inskeep and Bloom 1985) but, to the best of our knowledge, was not tested for Chlide

formation. This solvent prevents CLH activity even during an overnight incubation of *Arabidopsis* leaves at 4°C (Fig. 2-7). Therefore, the use of DMF appears to be the best option for extracting photosynthetic pigments from this model organism for downstream analysis using HPLC without introducing artifacts. However, this solvent is not as effective for *G. coronaria*, pea and cherry leaves as it is for *Arabidopsis* leaves (Fig. 2-8). Moreover, this solvent is a possible liver toxin (Wrbitzky 1999) and all appropriate safety guidelines should be adhered to in its use. Although the volatility of DMF is low, it should be carefully handled in an exterior venting fume hood. In conclusion, the use of DMF might be restricted to *Arabidopsis* or similar plant species in well-ventilated laboratory conditions.

2.5 Figures, Table and Legends

Table 2-1. Stability of chlorophyll *a* and chlorophyllide *a* after extraction with pure acetone. Mature Arabidopsis leaves (7th - 9th leaves counting from the bottom of the 4-week-old plants) were treated with 50 μ M MeJA and were kept in complete darkness for 3 days. Chlorophyll was extracted by immersing leaves in pure acetone as it is described in the "Time-course experiments" subsection of the Methods section. "Time" indicates the incubation time at room temperature. After removing the residual leaf tissue by centrifugation, the extracts were incubated for 24 hours at room temperature. Each line represents a single experiment with an extract from a single leaf, and experiments were repeated three times in the same conditions. Values represent chlorophyllide *a* or chlorophyll *a* concentrations per gram fresh weight of leaves. Chl *a*, chlorophyll *a*. Chlide *a*, chlorophyllide *a*.

0 day			1 day	
time	Chlide <i>a</i>	Chl <i>a</i>	Chlide <i>a</i>	Chl <i>a</i>
(min)	(nmol/gFW)	(nmol/gFW)	(nmol/gFW)	(nmol/gFW)
0	9	1284	9	1317
	5	1107	7	1167
	8	1338	6	1340
6	211	979	203	983
	191	1249	203	1329
	168	1052	171	1081

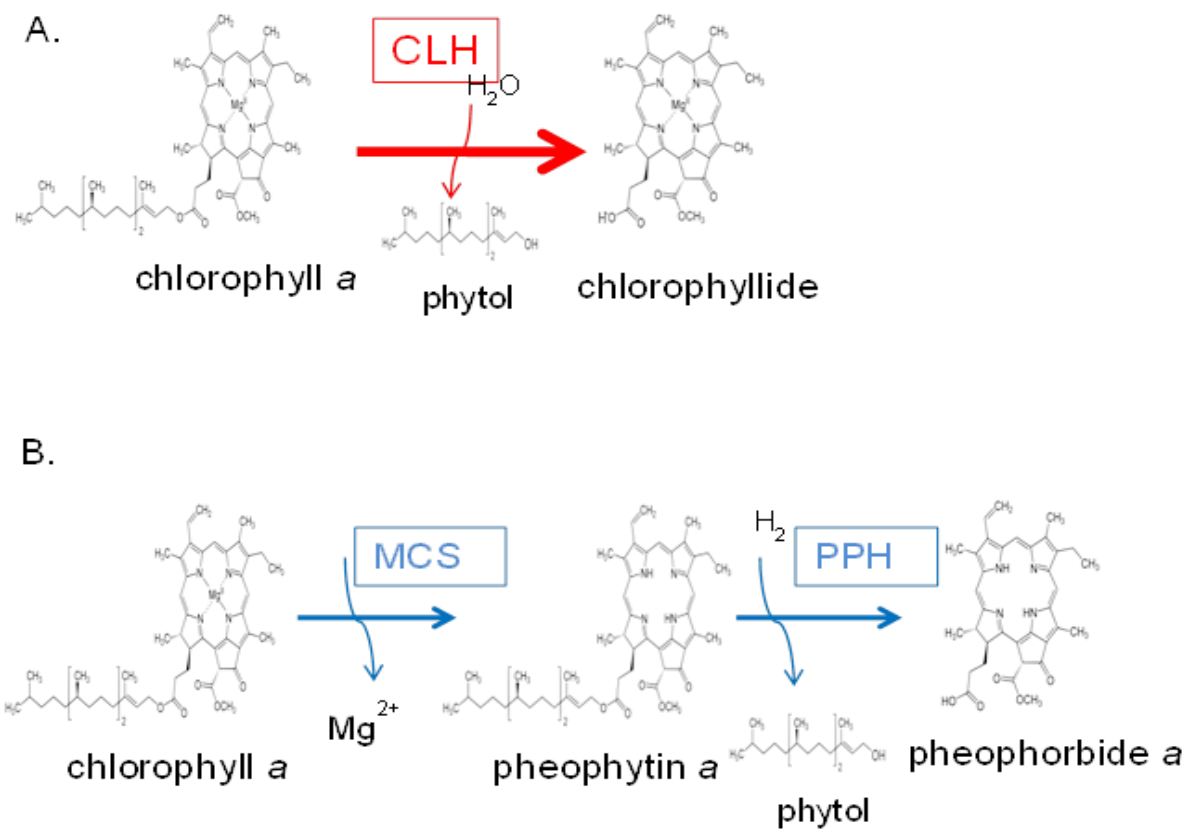


Figure 2-1

Figure 2-1. Comparison of the CLH reaction and a proposed *in vivo* degradation pathway of chlorophyll *a*. A. CLH catalyzes the hydrolysis of the ester bond of chlorophyll to form chlorophyllide and phytol. B. An *in vivo* degradation pathway of chlorophyll *a* proposed by Hörtensteiner and coworkers (Schelbert et al. 2009). MCS denotes magnesium dechelating substances. PPH denotes pheophytinase.

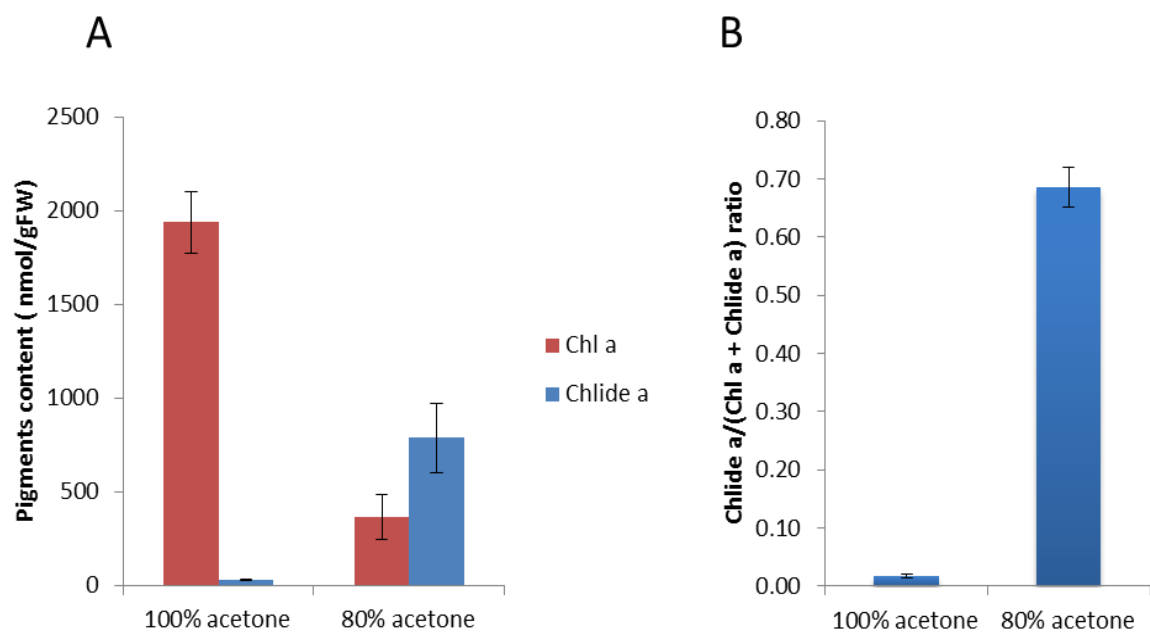


Figure 2-2

Figure 2-2. Formation of chlorophyllide *a* by the extraction of chlorophyll with pure or 80% buffered acetone. Frozen leaves were immersed in pure acetone or 80% acetone containing 20% (v/v) Tris-HCl, pH 8 and then incubated in these solvents for 12 h at 4°C in the dark. After that the pigments were extracted by grinding with stainless steel beads as described in the Methods section. A. Levels of chlorophyll *a* and chlorophyllide *a* per gram fresh weight of leaves. B. Chlorophyllide *a* levels in sample extracts expressed as the ratio of chlorophyllide *a* to the sum of chlorophyll *a* and chlorophyllide *a*. Chl *a*, chlorophyll *a*. Chlide *a*, chlorophyllide *a*. Error bars indicate standard deviations. Sample size, n = 3.

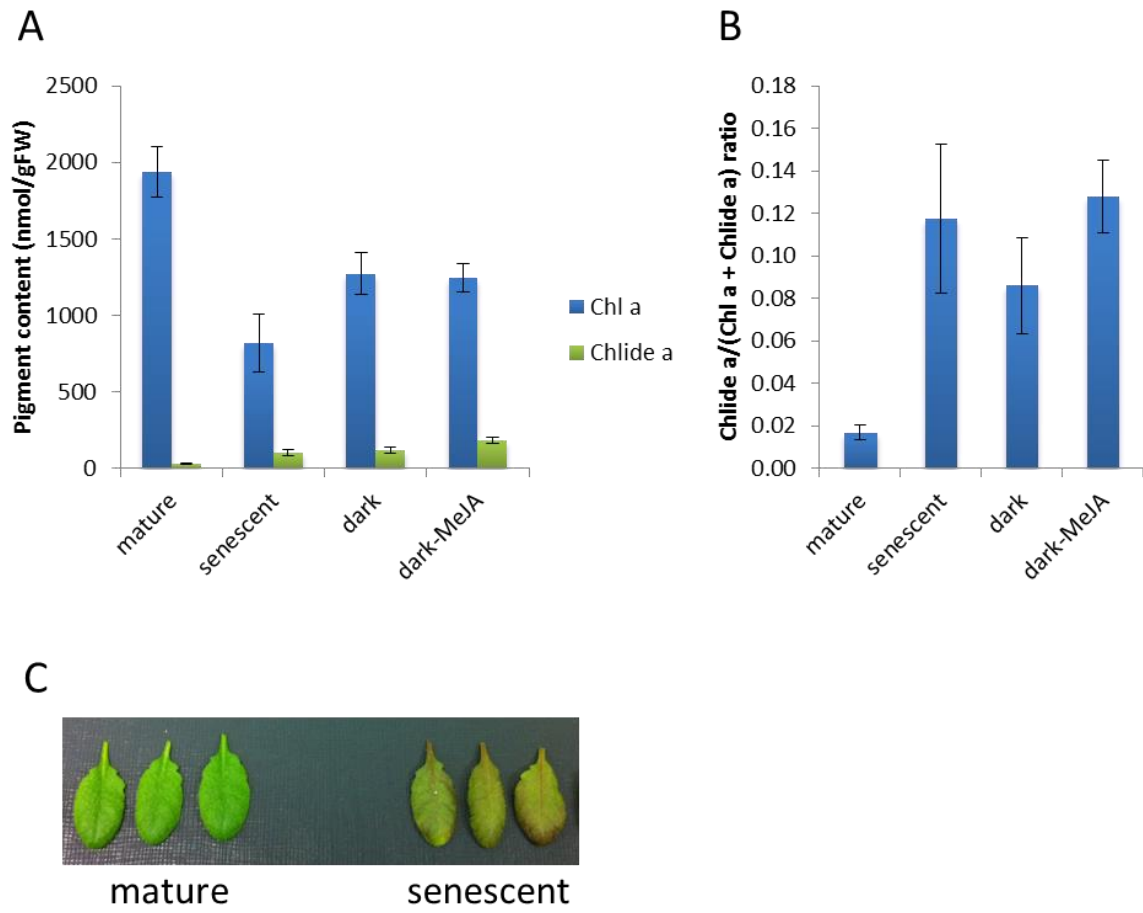
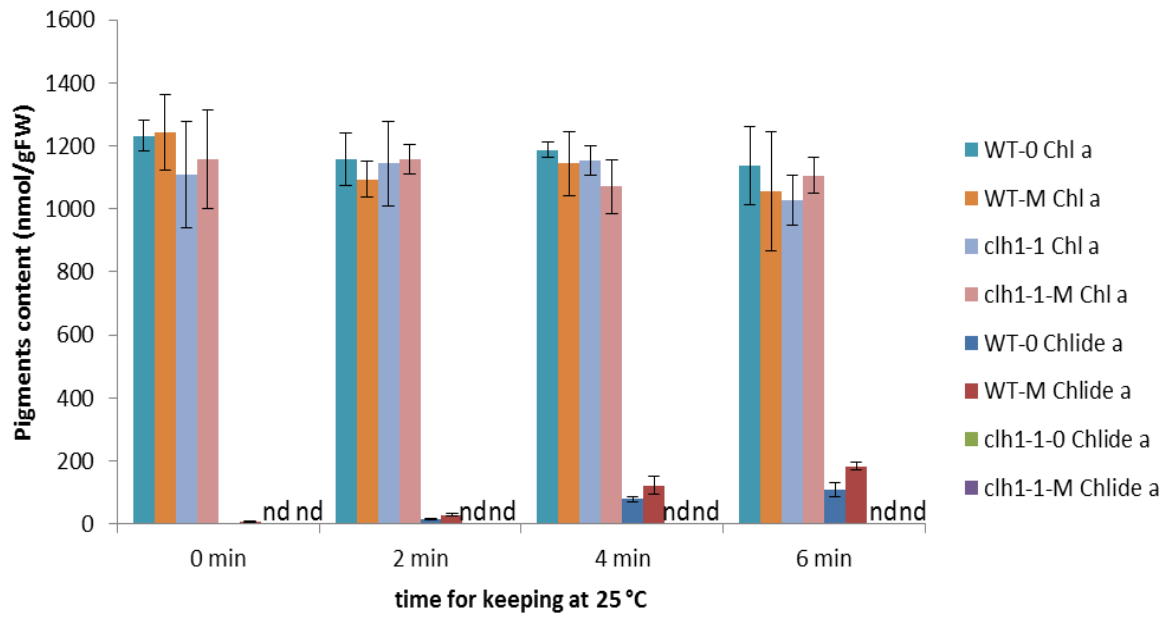


Figure 2-3

Figure 2-3. Chlorophyllide formation during pigment extraction from senescent or MeJA-treated leaves. Pigments extracted from mature leaves (7th to 9th leaves counting from the bottom of the plant) of wild-type (WT) *Arabidopsis* plants under four different conditions: leaves collected from 4-week-old plants ("mature"), leaves from 8-week-old plants ("senescent"), leaves from 4-week-old plants where the removed leaves were incubated in complete darkness on filter paper saturated with 3 mM MES buffer ("dark") or the same buffer plus 50 μ M MeJA ("dark-MeJA"). Pigments were extracted by immersing the leaves in pure acetone that was cooled to 4°C and subsequently analyzed by HPLC (see the Methods section for detail). A. Levels of chlorophyll *a* and chlorophyllide *a* per gram fresh weight of leaves. B. Chlorophyllide *a* levels in sample extracts expressed as the ratio of chlorophyllide *a* to the sum of chlorophyll *a* and chlorophyllide *a*. Chl *a*, chlorophyll *a*. Chlide *a*, chlorophyllide *a*. Error bars indicate standard deviations. Sample size, $n = 3$. C. The photograph below the bar graphs illustrates the 4-week-old (left) and 8-week-old (right) leaves used in these analyses.

A



B

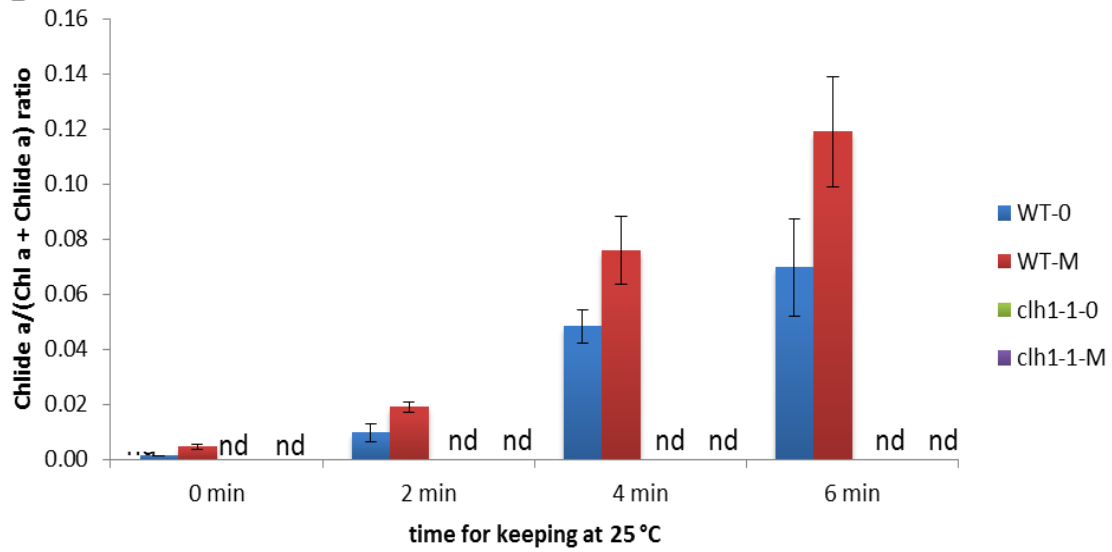
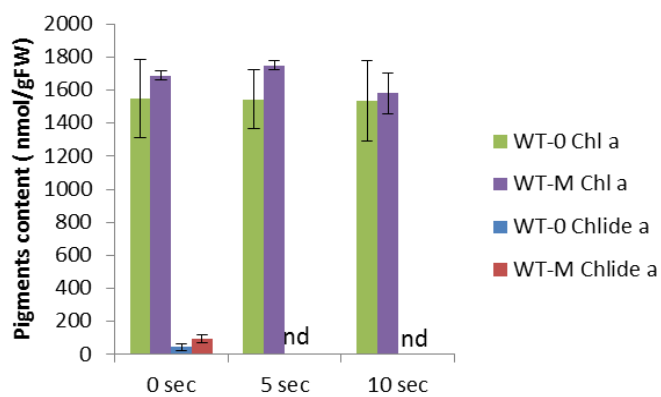


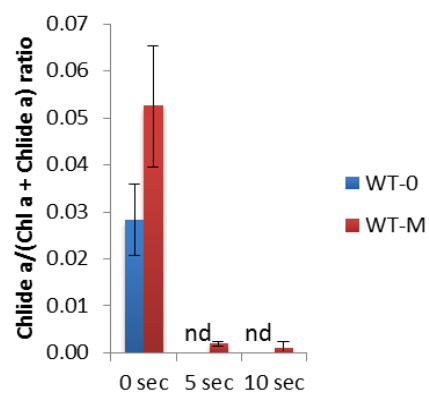
Figure 2-4

Figure 2-4. Time course of chlorophyllide *a* formation during pigment extraction. Chlorophyll was extracted by immersing leaves in pure acetone as it is described in the "Time-course experiments" subsection of the Methods section. A. Levels of chlorophyll *a* and chlorophyllide *a* per gram fresh weight of leaves. B. Chlorophyllide *a* levels in sample extracts expressed as the ratio of chlorophyllide *a* to the sum of chlorophyll *a* and chlorophyllide *a*. Chl *a*, chlorophyll *a*. Chlide *a*, chlorophyllide *a*. Error bars indicate standard deviations. Sample size, $n = 3$. n.d. = not detected.

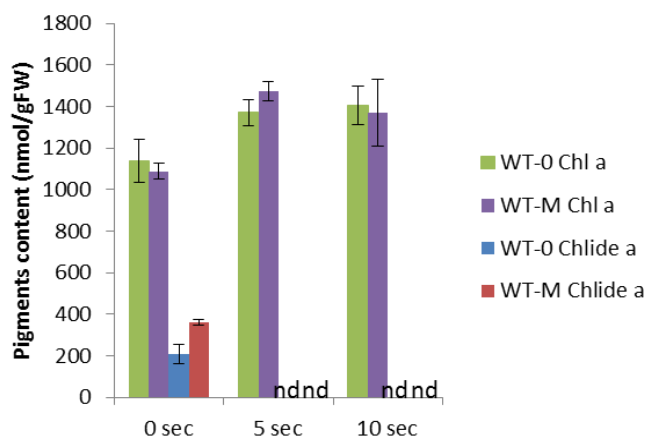
A



B



C



D

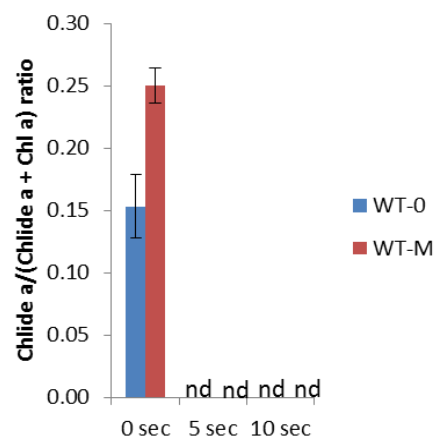


Figure 2-5

Figure 2-5. Effect of quick boiling on the formation of chlorophyllide *a* during extraction. Mature leaves (7th to 9th leaves counting from the bottom of the plant) were collected from 4-week-old wild-type plants that had been sprayed with 100 μ M MeJA and kept for 3 days at the same growth conditions. The collected leaves were immersed in pure acetone at room temperature directly or were boiled for 5 and 10 sec respectively before they were immersed in pure acetone, then they were ground with stainless steel beads by vigorous shaking (A and B). Alternatively, pigments were extracted by immersing leaves in pure acetone at 4°C for 12 hours (C and D). A and C, Levels of chlorophyll *a* and chlorophyllide *a* per gram fresh weight of leaves. B and D, Chlorophyllide *a* levels in sample extracts expressed as the ratio of chlorophyllide *a* to the sum of chlorophyll *a* and chlorophyllide *a*. Chl *a*, chlorophyll *a*. Chlide *a*, chlorophyllide *a*. Error bars indicate standard deviations. Sample size, $n = 3$. n.d. = not detected.

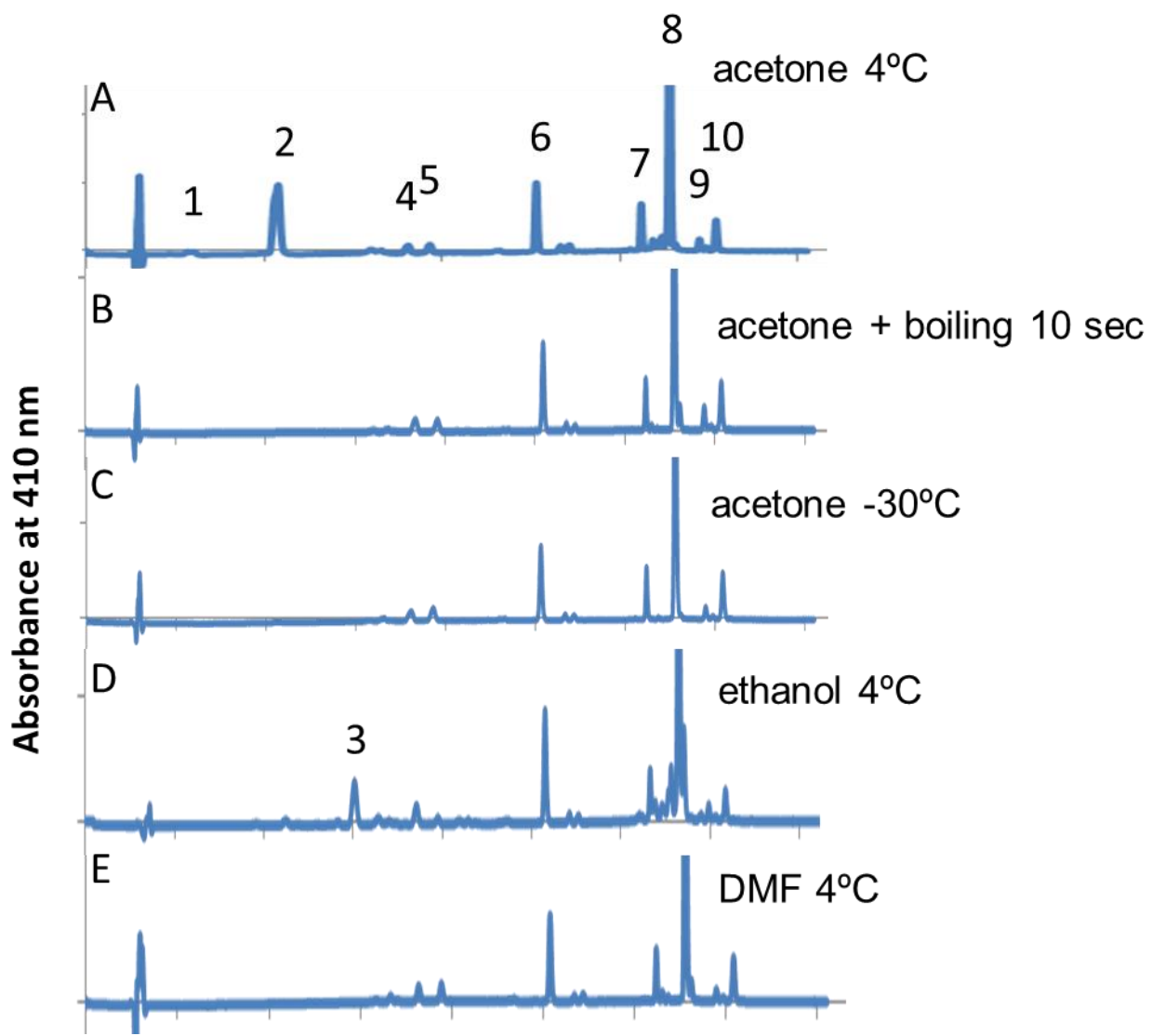


Figure 2-6

Figure 2-6. The elution profiles of pigments from extracts separated by HPLC.

Representative HPLC profiles from each experiment are shown. A. HPLC profile of pigments extracted by immersing wild-type (WT) leaves in pure acetone for 12 hours at 4°C. B. An HPLC profile of WT pigments extracted by the quick boiling method. C. HPLC profile of pigments extracted from leaves of WT plants by immersing leaves in pure acetone for 96 hours at -30 °C. D. HPLC profile of pigments extracted from leaves of WT plants by immersing leaves in ethanol at 4°C for 12 hours. E. HPLC profile of pigments extracted from leaves of WT plants by immersing leaves in DMF at 4°C for 12 hours. Peak 1, chlorophyllide *b*. Peak 2, chlorophyllide *a*, Peak 3, pheophorbide *a*. Peak 4, neoxanthin. Peak 5, violaxanthin. Peak 6, lutein. Peak 7, chlorophyll *b*. Peak 8, chlorophyll *a*. Peak 9, pheophytin *a*. Peak 10, β -carotene.

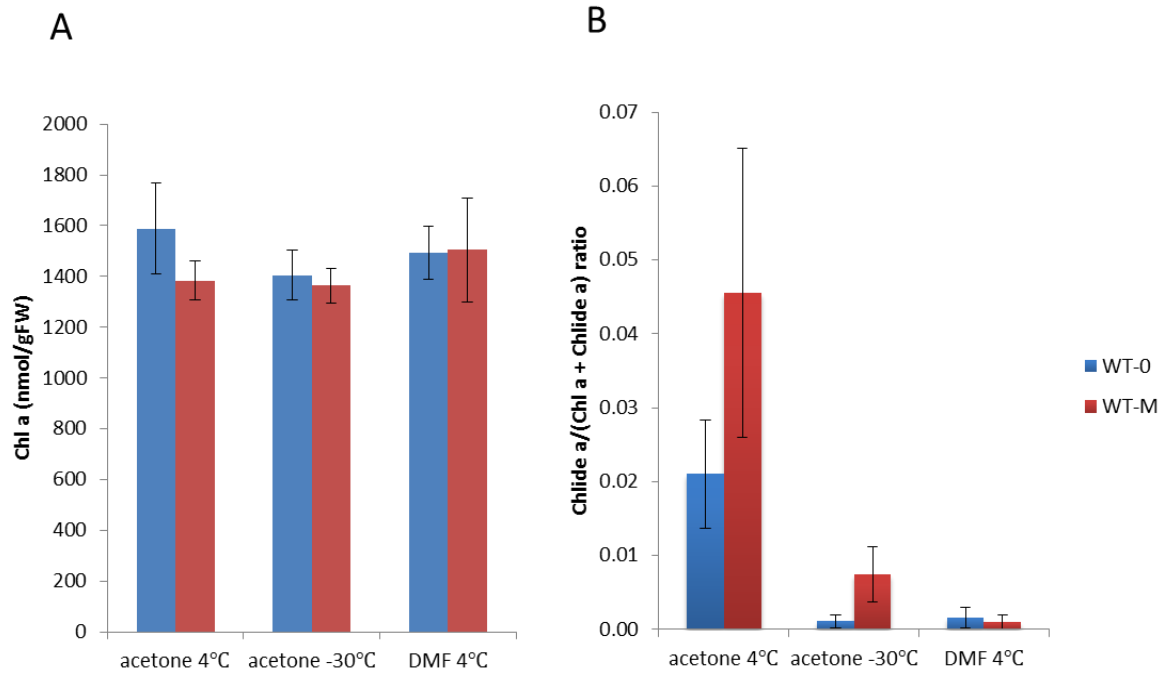


Figure 2-7

Figure 2-7. Pigment extraction at -30°C in pure acetone and at 4°C in DMF.

Mature *Arabidopsis* leaves (7th to the 9th leaves counting from the bottom of the plant) that were treated with or without MeJA (see the Method section for the detail) were harvested and their pigments were extracted by three different methods. In the first two methods, leaves were immersed in pure acetone at 4°C for 12 hours ("acetone 4°C") and at -30°C for 96 hours ("acetone -30°C") respectively. In the third method, leaves were immersed in DMF at 4°C for 12 hours ("DMF 4°C"). A. Levels of chlorophyll *a* and chlorophyllide *a* per gram fresh weight of leaves. B. Chlorophyllide *a* levels in sample extracts expressed as the ratio of chlorophyllide *a* to the sum of chlorophyll *a* and chlorophyllide *a*. Chl *a*, chlorophyll *a*. Chlide *a*, chlorophyllide *a*. Error bars indicate standard deviations. Sample size, $n = 3$. n.d. = not detected.

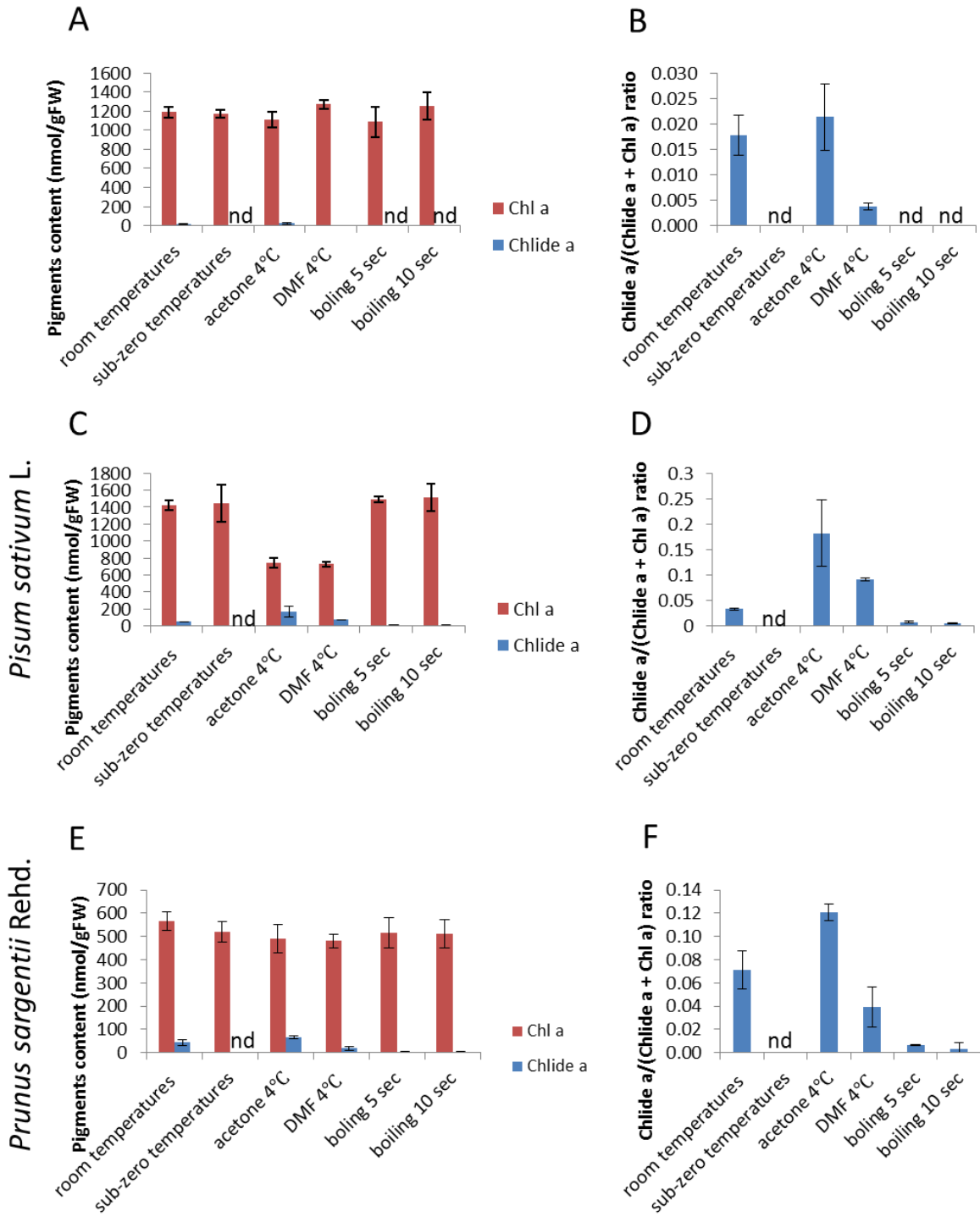


Figure 2-8

Figure 2-8. Comparison of extraction methods for chlorophyllide formation with three different species (*Glebionis coronaria*, *Pisum sativum* L. and *Prunus sargentii* Rehd.). Leaves were homogenated in acetone at room temperatures ("room temperatures") or sub-zero temperatures ("sub-zero temperatures"), respectively, by grinding leaves with steel stainless beads. Alternatively, leaves were immersed in pure acetone at 4°C ("acetone 4°C") or in pure DMF at 4°C ("DMF 4°C"), respectively, without the grinding procedure. In the last pair of experiments, leaves were boiled for 5 ("boiling 5 sec") or 10 sec ("boiling 10 sec"), and then they were immersed in acetone at 4°C for chlorophyll extraction. The detail of the methods is described in the Methods section. A, C and E. Levels of chlorophyll *a* and chlorophyllide *a* per gram FW of leaves. B, D and F. Chlorophyllide *a* levels in sample extracts expressed as the ratio of chlorophyllide *a* to the sum of chlorophyll *a* and chlorophyllide *a*. Chl *a*, chlorophyll *a*. Chlide *a*, chlorophyllide *a*. Error bars indicate standard deviations. Sample size, n = 3. n.d. = not detected.

**CHAPTER 3: PLANTS USE CHLOROPHYLL-
DERIVATIVES FOR DEFENSE AGAINST
INSECT HERBIVORES THROUGH CLH
ACTIVITY**

ABSTRACT

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 also can be used for defense against insect herbivores are unknown. Chlorophyllase (CLH), which catalyzes the release of the phytol chain from 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., 2007). 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 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). We subsequently demonstrated that Chlide and

pheophorbide (Pheide) were poisonous to the larvae of a common generalist *Spodoptera litura*. In addition more larvae were killed by feeding the two Chl-derivatives, and the development of the larvae was significantly delayed by feeding Chlide. In contrast, 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, the leaves were homogenized under either pH8 or pH10 conditions. The Chlide amount was subsequently analyzed by HPLC after the leaf homogenate was incubated at room temperature. The result showed that much Chlide were produced in the leaves mixture of CLH1 overexpression lines and WT and only little in *clh1-1* mutant. Finally we fed *Spodoptera litura* with WT, *clh1-1* and three CLH1 overexpression lines for 11 days. Although almost all of the larvae who ate WT and *clh1-1* were survived, the average survival rates were significantly decreased when they ate the leaves of three CLH1 overexpression lines. Taken together, we conclude that CLH1 is not involved in Chl breakdown even during MeJA-induced senescence in Arabidopsis. We suggest that plants utilize 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.

Keywords: Chlorophyllase; Methyl jasmonate; Chlorophyll-derivatives; Insect herbivore; Defense

3.1 Introduction

Plants suffer from abiotic and biotic stress throughout their life in their natural environment because they are sessile organisms. Except the competition among plants, herbivores and pathogens are the main biotic stresses. As one of the most important group, insect herbivores have been living together with plants for more than 350 million years (War et al. 2012) (Fürstenberg-Hägg et al. 2013). Although the size of each insect herbivore is small, the populations and types are amazing. Losses due to insect herbivores were estimated at 10-20% of major crops (Ferry et al. 2004). Especially in some seasons or years when the natural conditions are quite suitable for the growth of insect herbivores, they will become to a disaster to plants. For example, plague development have occurred with the locust in many countries and regions in the world (Showler 1995) (Z Liu et al. 2008). On the other hand, plants also developed defense systems to protect themselves from insect herbivores. Fully utilizing the defense system which produced by plants themselves was one of the effective and environmentally-friendly strategy to protect our target plants, especially crops. For example, maize was protected from lepidopteran larvae by the co-cultivation with an Africa grass (*melinis minutiflora*) that release abundant volatile compounds (Z.R. Khan et al. 1997) (Z.R. Khan et al. 2000). Therefore, studying on the mechanism of plant defense against insect herbivores not only increase our base knowledge of science, but also help us to control the pest

environmentally sustainable and cost-effective by improving plant resistant other than by spraying pesticide.

The types of plant defense against insect herbivores can be divided into two large groups: direct and indirect defense reactions. Direct defense also contains two groups: structural and chemical defense. Structural defenses are usually barriers trait for insect herbivores such as sclerophylly and pubescence (Agren and Schemske 1993) (Hanley et al. 2007). Chemical defenses, except some heavy metals such as zinc, lead and nickel can be concentrated from the environment by some plant species for defense (Jhee et al. 2005) (R Boyd 2012), organic compounds for defense are well reported (Mithöfer and Boland 2012). They almost cover all classes of (second) metabolites which are produced by plants in order to retard the normal growth of insect herbivores (I Baldwin and Preston 1999) (I Baldwin 2001). These compounds belong to various chemical classes such as isoprene-derived terpenoids, N-containing alkaloids, phenolic compounds and so on (Mithöfer and Boland 2012). In addition, even amino acids, peptides and fatty acid derivatives are used by plants for defense as well (Hylin 1969) (Ryan and Byrne 1988) (T Huang et al. 2011) (Habib and Fazili 2007). Some of the defense compounds specially exist in some plant species, while others ubiquitously exist in almost all of the plants as common defense systems.

Instead of direct defenses, indirect defenses are that plants reduce insect herbivores by increase predation of herbivores by luring and keeping predators on a plant with food rewards, shelters from harsh conditions, or chemicals signaling prey availability. The only indirect defenses that actively attract predators are volatile organic chemicals (VOCs) (Mortensen 2013).

Some of the defenses are constitutive (pre-formed), while others are induced after attack by insect herbivores (Wittstock and Gershenzon 2002) (M Chen 2008). Inducible defenses incur less metabolic costs to host plants because they only induced when insect attack happen. Inducible defenses were divided into three steps: surveillance, signal transduction, and the production of defensive chemicals (A Kessler and IT Baldwin 2002) (Ferry et al. 2004) (Smith and Boyko 2007). Although there are many signal transduction pathways for defense against insect herbivores in plants, in general, a phytohormone – jasmonates (JA) are regarded as important signalling substances upon wounding or biotic attack (Creelman and Mullet 1997). The JA signalling pathway is important for defense against necrotrophic pathogens and also against insect herbivores, although the crosstalk of JA with other two phytohormones: salicylates and ethylene also exist in plants (KL-C Wang et al. 2002). Many plant defense genes are inducible though JA pathway such as *VSP2* and *PDF1.2* (Pieterse et al. 2009).

Chlorophyll (Chl), the most abundant pigment on earth, is an essential component in photosynthesis. It is responsible for harvesting solar energy in photosynthetic antenna systems, and for charge separation and electron transport within reaction centers. However, Chl is also a dangerous molecule and a potential cellular phototoxin. For example, Chl and its derivatives are strong photosensitizers; that is, when the present is excess, they will generate reactive oxygen species (ROS) (Camp et al., 2003). As it allows for recycling of nitrogen and other nutrients for protection from buildup of phototoxic Chl intermediates (reviewed in Hörtensteiner, 2006), Chl degradation is vital during leaf senescence or fruit ripening (Kräutler 2008), but also as a response to many biotic and abiotic stresses (Benedetti and Arruda 2002) (Kariola et al. 2005) (Hörtensteiner and Kräutler 2011). Chl metabolism is highly regulated during plant development. However, Chl degradation mechanism is less understood than that of Chl biosynthesis. A Chl breakdown pathway had been suggested based on the identification of key enzymes and catabolites along the pathway. In this pathway, removal of the phytol chain by chlorophyllase (CLH) is the first step, which converts Chl to phytol and chlorophyllide (Chlide). Then after Mg-dechelation from Chlide, the porphyrin ring of pheophorbide (Pheide) is oxygenolytically opened by Pheide *a* oxygenase (PAO) inside of chloroplast, resulting in loss of the green color of the molecule. After that, the intermediate product, Chl catabolite (RCC) is degraded by reductase

(RCCR). The product, a primary fluorescent catabolite (pFCC), is transported to the vacuole and converted to the final non-fluorescent Chl catabolites (NCCs) by non-enzymatic tautomerization (Hörtensteiner, 2006) (Hörtensteiner and Kräutler 2011).

CLH was discovered about 100 years ago (Stoll, 1912). In vitro, this enzyme displays high activity to catalyze the hydrolysis of Chl to phytol and Chlide (Stoll, 1912). Afterwards, many researchers detected this enzyme's activity and analyzed the catalytic characterization of it in algae and green tissue of many plants (Ardao and Vennesland, 1960) (Mcfeeters et al., 1971) (Klein and Vishniac, 1961) (Terpstra and Lambers, 1983) (Benedetti and Arruda, 2002) (Arkus et al., 2005) (Shioi et al. 1991). Therefore, for a long time, CLH has been considered the first enzyme involving in the progress of Chl degradation (Hörtensteiner, 2006). However, whether this enzyme is involved in Chl breakdown in vivo was questioned recently, based on the research using *Arabidopsis* mutants in which CLHs was absent. In *Arabidopsis*, there are two isoforms of CLH (CLH1 and CLH2). Interestingly, both *clh1* and *clh2* single and double knockout lines were still able to degrade Chl during dark-induced leaf senescence (Schenk et al., 2007), which suggested that both CLH1 and CLH2 are not essential for dark-induced leaf senescence-related Chl breakdown in *Arabidopsis*. Instead, another enzyme, which was named as pheophytinase (PPH) was localized to the chloroplast and the PPH

knock-out lines showed a strong stay-green phenotype during dark-induced leaf senescence (Schelbert et al., 2009). They found PPH possess the activity to catalyze the conversion of pheophytin to Pheide, while it cannot catalyze the conversion of Chl to Chlide although both reactions regard to removing the phytol chain. Therefore, another Chl degradation pathway, PPH pathway should exist in plants. PPH pathway starts with removing Mg^{2+} from Chl *a* by metal-chelating substance, then the phytol side chain is removed from the last step product pheophytin *a* to produce Pheide *a* by the catalyzation of PPH (Schelbert et al., 2009). Schelbert and coworkers suggested that in dark-induced senescence, PPH pathway takes charge of Chl degradation rather than the CLH pathway. As well as known, under normal growth condition, Chl degradation in plants happened only in the old leaves because of the development stage, while Chl degradation will be started earlier and faster in leaves when plants are under stress or phytohormones treated conditions. The age dependent and independent senescence mechanisms are largely different with each other (Weaver et al. 1998) (BF Quirino et al. 2000). Therefore, it is possible that the Chl degradation pathway also different between the two types of Chl degradation. It was reported that *CLH1* mRNA was induced rapidly by a treatment of Methyl-jasmonate (MeJA), wounding, pathogen infection and other stress conditions (Benedetti et al. 1998)(Kariola et al. 2005) while *CLH2* did not respond to MeJA (Tsuchiya et al., 1999). Because MeJA is a well-known

phytohormone promoting senescence and Chl degradation in plants (He et al. 2002), it could be hypothesized that CLH1 was important for Chl breakdown specially during MeJA (or other stress)-induced senescence and Chl catabolism in Arabidopsis like citrus CLH was involved in ethylene-induced fruit color – break (Azoulay Shemer et al. 2008).

Intracellular localization is often a key strategy to understand protein functions and regulation in vivo and has been a controversial issue with regard to the enzyme CLH (Azoulay Shemer et al., 2008). A lot of research reported that CLHs from different kinds of plant were located in chloroplasts (Krossing, 1940) (Garcia and Galindo, 1991) (Matile et al., 1997) (Trebitsh et al., 1993) (Okazawa et al., 2006). Even until recently, it was reported that both the precursor and mature forms of citrus CLH was located in chloroplasts during ethylene-induced Chl breakdown (Harpaz-Saad et al., 2007) (Azoulay-Shemer et al., 2011) (Azoulay Shemer et al., 2008). However, after cloning of the two isoforms of CLH, no typical feature of signal sequence for chloroplast targeting was identified in CLH1, whereas CLH2 had only a weakly predicted signal sequence for chloroplasts (Tsuchiya et al., 1999). In Arabidopsis, CLH1 is the major isoform and it contributes to the most activity of CLHs (Schenk et al., 2007). It was hypothesized that CLH1 was located outside of chloroplast, and there was an additional Chl degradation pathway located outside of chloroplast (Takamiya et al. 2000). In additionally, transient

expression of CLH1-YFP (C-terminal fused) in Arabidopsis protoplast showed that CLH1 was located in cytosol rather than chloroplast (Schenk et al., 2007). One reasonable explanation was that the transient expression system was not stable for the existence of the fusion protein, the GFP signal could not response the true localization of CLH. The other explanation was suggested that GFP fusions may interfere with the natural localization of CLHs due to masking of amino- and carboxy-terminal targeting and modification signals (Tian et al., 2004) (Azoulay Shemer et al., 2008). Therefore, to identify the localization of CLH1, it was necessary to check the localization of CLH1-YFP which was stably overexpressed in plants. Furthermore, the localization of native CLH1 is also required.

In this study, we aimed at elucidating what is the function of CLH. By observation the phenotype of single and double knockout mutants of CLHs and wild type (WT) during MeJA-induced senescence, and also the localization of CLH1, our data showed that Arabidopsis CLH1 is not involved in Chl breakdown during MeJA-induced senescence in Arabidopsis. However, we demonstrate that Chlide and Pheide are poisonous to the larvae of a common generalist *Spodoptera litura*. After the leaves were eaten by insect herbivores, Chlide can be produced during chewing and digestion. The amount of Chlide formation heavily dependent on the activity of CLH. Finally we employed WT, *clh1-1* and three CLH1 overexpression lines to test the same kind of insect larvae. Although almost all of the larvae who ate WT

and *clh1-1* were survived, the average survival rate of larvae was significantly decreased when they ate the three CLH1 overexpression lines. Taken together, we conclude that CLH1 is not involved in Chl breakdown even during MeJA-induced senescence in Arabidopsis. Here, we would like to suggest that 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.

3.2 Materials and methods

3.2.1 Plant materials

Arabidopsis T-DNA insertion lines SALK_124978 (designated *clh1-1*; ecotype Columbia (col)), SAIL_646_E09 (designated *clh2-2*; ecotype col), *clh1-1/clh2-2* were described by Schenk et al. (Schenk et al., 2007). Another T-DNA insertion line GK-453A08 (designated *pph3*; ecotype col) was obtained from the European Arabidopsis Stock Center, Nottingham, UK (Schelbert et al. 2009). GFP::cDNA transgenic lines (Q4 (GFP-ER protein) and Q5 (GFP- δ -TIP)) were obtained from The Arabidopsis Information Resource (TAIR, (Cutler et al. 2000)). Homozygous plants were identified by PCR using T-DNA and gene-specific primers as listed by Schenk et al. and Schelbert et al. (Schenk et al., 2007) (Schelbert et al., 2009).

Plants were grown on soil in long-day (16 h light/8 h dark) or short-day (10 h light/14 h dark) growth conditions under fluorescent light of 70–90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 23 °C for 4 weeks. For senescence, all the leaves from each plant which was grown in long day condition were detached and placed on wet filter paper (3 mM MES buffer, pH 5.8, with or without 50 μM MeJA) and incubated in permanent darkness for up to 4 days.

3.2.2 Pigment analysis

The leaves were imaged by digital camera, and the sizes were calculated by ImageJ software (<http://rsbweb.nih.gov/ij/>). Pigments were extracted from leaf tissue by homogenization at -20°C with acetone (Hu et al. 2013). Extracts were centrifuged for 5 min at 15,000 rpm at 4°C, and the supernatant was analyzed by HPLC with Symmetry C8 column (150 mm in length, 4.6 mm in i.d.; Waters, Milford, MA, USA) according to the method of Zapata et al. (Zapata et al., 2000). Elution profiles were monitored by measuring absorbance at 410 nm. Standard pigments (Chl *a* and Chl *b*) were purchased from Juntec Co. Ltd. (Odawara, Japan).

3.2.3 Arabidopsis transformation and confocal microscopic analysis of the localization of eYFP

Complementary DNA of CLH1 was cloned from the wild-type plants. eYFP was cloned from pEarleyGate101 plasmid (Earley et al., 2006). For C-terminal fusion

(CLH1-eYFP), the construct was generated by fusing the eYFP sequence to the C terminus of CLH1, and then 5'UTR and 3'UTR of CLH1 were fused to the N and C-terminal of the fusion sequence respectively. By performing the LR clonase reaction, the construct cloned in pENTR was transferred into the expression vector pEarleyGate100 (Earley et al., 2006) containing a gene conferring glufosinate resistance (Nakagawa et al., 2007) under the control of the 35S promoter of pBI221 (Clontech). The recombinant binary vector was introduced into the *Agrobacterium* (*Rhizobium radiobacter*) strain GV2260. *Agrobacterium* was infected into *Arabidopsis* using the floral dip method (Clough and Bent, 1998). Two-week-old seedlings of transformants were selected by spray basta.

Protoplasts were isolated from mature leaves of 4-old-week plants by following the method described by Robert and coworkers (Robert et al., 2007). Expression and localization of the CLH1-eYFP protein in transformed transgenic plants were examined under the confocal fluorescence microscopy (describe instrument, Nikon Co. Ltd., Japan).

3.2.4 Preparation of membrane and soluble fractions

Leaves from 4-week-old plants grown under the short-day conditions (short day) were harvested and lysed using a mortar and pestle under liquid nitrogen. The fractions were prepared as described by Oka et al. (Oka et al., 2010).

3.2.5 Isolation intact chloroplasts and vacuole

Arabidopsis rosette leaves are harvested from 4-week-old Arabidopsis which were grown in the short-day conditions. Intact chloroplasts were prepared essentially according to the method that was described by Salvi et al. (Salvi et al., 2008). Intact vacuoles were prepared according to the protocol that was described by Robert et al. (Robert et al., 2007). Protein concentrations were determined by Lowry analysis ((Lowry et al., 1951), with bovine serum albumin (BSA) (Sigma Chemical) as standard protein.

3.2.6 Membrane fractionation

Arabidopsis leaves used for membrane fractionation were grown on soil for 28 days in the short-day conditions. The fractionation method was modified from which was described by Oka et al. (Oka et al., 2010) and Wulfetange et al. (Wulfetange et al., 2011). Leaf tissues was homogenized in buffer containing 50 mM Tris (pH 7.6 at 22°C), 20% glycerol, and 150 mM NaCl with either 5 mM MgCl_2 and 2 mM EGTA (for subsequent sucrose (Suc) gradient separation in the presence of Mg^{2+}) or with only 2 mM EDTA (for subsequent Suc gradient separation in the absence of Mg^{2+}). Leaves were homogenized three times at a low speed in a blender with a 10-fold amount of buffer. The homogenates were filtered through two layers of Miracloth (Merck) and then centrifuged for 10 min at 10,000 g at 4 °C. The supernatants were ultra-centrifuged for 30 min at 100,000 g at 4°C.

The resulting pellets were resuspended in 0.5 mL/g in buffer containing 50 mM MOPS-NaOH, PH 7.0, 10% (w/v) Suc, EDTA-free complete protease inhibitor cocktail (Sigma), and either 5 mM or 5 mM $MgCl_2$. Then the suspension was centrifuged at 6,000 g for 3 min to remove un-suspended small pellets. Six hundred ml of the supernatant was loaded onto the top of the following discontinuous Suc gradient: from bottom to top, 200 μ l of 55% Suc, 450 μ l of 50%, 45%, 40%, 35%, 30%, 25% and 20% Suc solution containing the same components with the re-suspension buffer except Suc. Then the gradient was centrifuged in a bucket rotor for 20 h at 100,000 $\times$ g at 4°C. After centrifugation, the gradient was collected in 200 μ l increment fractions from top to bottom, and the fractions were stored at -30°C before using.

3.2.7 Immunoblot analysis

Total protein was extracted from leaves using extraction buffer containing 50 mM Tris-HCl (pH=8), 12% Sucrose, 2% LDS, and 1.5% DTT. Before SDS-PAGE separation, all the samples were mixed with equal volume of 2 $\times$ Ling's urea buffer containing 8 M urea, 30 mM Suc, 10 mM Tris-HCl, pH 8.0, 40 mM dithiothreitol, 2% SDS, 1 mM EDTA and 10 μ g/mL Pyronin Y, and then they were separated on a 14% polyacrylamide gel and electroblotted to PVDF membrane. The CLH1 protein was detected with an anti-CLH1 antiserum (rabbit) using the ECL Plus immunodetection kit (GE Healthcare). The membrane type was identified by the

use of a set of polyclonal rabbit antibodies against specific markers: ER luminal BiP2 (Agrisera), Plasma membrane (PM) marker H⁺-ATPase (Agrisera), tonoplast marker V-PPase (CosmoBio, Inc.). Thylakoid membranes were identified by HPLC analysis of the Chl content (Zapata et al., 2000).

3.2.8 CLH Assay

The method for CLH assay was modified from which was described by Tsuchiya and coworkers (Tsuchiya et al., 1997). Leaves were homogenized with 10 times weight of the assay buffer containing 50 mM Tris-HCl, 38 mM octylglucoside (OG), 1% (v/v) protease inhibitor cocktail (PH=7.5, on ice). The homogenate was centrifuged for 10 min at 15,000 rpm at 4 °C. The supernatant was transferred into a 1.5 mL microcentrifuge tube. The pellet was re-extracted with 10 times weight of the assay buffer, and the extract was pooled in the same 1.5 mL microcentrifuge tubes. To 375 µl of an extract (or buffer only as the control), 125 µl of acetone containing Chl *a* (500 µg/ml) was added and incubated for 10 min at 37°C. The reaction was stopped by adding 1 ml of hexane and 0.5 ml of acetone to the reaction mixture. Then the total mixture was vigorously shaken and centrifuged for 5 min at 10,000 ×g at 4°C, so that the Chlide *a* by the enzyme reaction was partitioned into the lower aqueous layer. After centrifugation at 10,000 × g for 5 min, Chlide was partitioned into the lower aqueous acetone layer, so that the lower layer was recovered to a new tube. The concentrations of Chlide *a* was determined

by measuring an absorbance at 667 nm using the absorption coefficients of 76.79 mM⁻¹cm⁻¹ for Chlide *a* (Porra et al., 1989). Chlide *a* amount which was produced during a unit time was defined as the activity of CLH. The relative activity of CLH in 50 µM MeJA treated leaves for 2 days was considered as 100%.

3.2.9 Pigments preparation

Pheide *a* was purchased from Wako Pure Chemical Industries, Ltd. Japan.

For preparing Chlide, the method was modified from the Müller and coworkers' method (AH Müller et al. 2011). *Glebionis coronaria* (garland chrysanthemum) leaves was purchased from the supermarket in Sapporo, Japan. Leaves of *G. coronaria* were ground in liquid nitrogen using mortar and pestle. The fine powder (5 – 10 g) was suspended in 20 ml of acetone, and then 20 ml of Tricine – NaOH (0.2 M, pH 8.0) was added. After shaking at 220 min⁻¹ for 2 h at room temperature in the dark, the leaf debris was removed by centrifugation and the green supernatant was repeatedly extracted with 40 ml of hexane until the epiphase was colorless. The leaf debris can be used for extracting Chlide additional twice following the same procedure. Diethyl ether was added twice to the pigment solutions, and the green epiphases were combined and dried in N₂.

For preparing Chl, the fine leaf powder was suspended in the same volume of acetone, first shaking at 220 min⁻¹ for 2 h at room temperature in the dark, then 20

ml of Tricine – NaOH (0.2 M, pH 8.0) was added. The leaf debris was removed by centrifugation and the green supernatant was extracted with 40 ml of hexane, then pigments appeared in the hexane phase were collected and dried in N₂.

Before HPLC purification, small amount of pure acetone was added to the dried pigments in order to dissolve all of the pigments. For HPLC purification, C8 column and solvent A and B of mobile phase which was reported by Zapata and coworkers were used (Zapata et al. 2000). Different gradient profiles were adjusted for separating Chlide *a* and Chl *a* (Table 3-1). For purification Chlide, The flow rate was fixed at 9 ml min⁻¹.

After getting the target pigments in the HPLC solvent, Diethyl ether and water were added immediately to concentrate target pigments into the Diethyl ether phase. After the green epiphases were combined, small volume of green epiphases was taken out and dried in N₂. Then 80% acetone (acetone:water (v:v) =8:2) was added to dissolve the pigments, and then the concentration was analyzed by spectrophotometer (Porra et al. 1989). Later the total amount of pigments was calculated. Finally, all of the green epiphases were dried in N₂ and stored in deep freezer.

3.2.10 Pigments feeding experiments

Pigments were first dissolved into the same amount of DMF, then mixed with artificial food. The final concentration of DMF was 0.1% in food with 400 nmol/g Chlide *a*, 200 nmol/g Chlide *a*, 400 nmol/g Chlide *a*, 400 nmol/g Chl *a* respectively, and control without any pigment but same concentration of DMF. Each group including 40 *Spodoptera litura* larvae which were just hatched. The same amount of food mixed with different kinds or concentrations of pigments were fed to each larva which was separated from each other by plastic tubes. The growth status of each larva was recorded at 11th day.

3.2.11 Leaf homogenization experiment

CLH1-YFP were overexpressed in the background of *chl1-1*. Three stable overexpression lines, WT and *chl1-1* were used for this experiment. Plants were grown in soil under short-day conditions (10 h light/14 h dark) in growth chambers under fluorescent light (70-90 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) at 23°C. Each sample including 0.3 g mature fresh leaves from 7-week-old plants. They were subsequently homogenized by glass homogenizer with 1ml pH8 (Tris-HCl) or pH10 (0.1 M Na_2CO_3 - NaHCO_3) buffer respectively. Then the leaf mixture was kept at room temperature (25°C) for 0.5 h or 2 h. After incubation, pigments were extracted by pre-cooled pure acetone (-30°C) (Hu et al. 2013) and analyzed by HPLC using Zapata's method (Zapata et al. 2000).

3.2.12 Plant feeding experiment

The same three stable CLH1 overexpression lines, WT and *clh1-1* mutant were grown in soil under short-day conditions (10 h light/14 h dark) in growth chambers under fluorescent light (70-90 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) at 23°C. Twelve uniform plants from each plant line were fed by 60 *Spodoptera litura* larvae which were just hatched (each plant was eaten by 5 larvae). Then larvae growth and survival status were observed at 11th day.

3.3 Results

3.3.1 Both the expression levels of CLH1 and total CLH activity were induced by MeJA treatment.

It was suggested that Arabidopsis CLH1 expression levels were induced by MeJA treatment. However, these results were just showed at the transcription levels (Benedetti et al. 1998) (Tsuchiya et al. 1999). To demonstrate that CLH1 is really induced by MeJA at translation levels and also all the induced CLH1 is active, the following experiments were performed.

Firstly, the specificity of the antibody for CLH1 was checked by comparing the western-blotting signals between WT and *clh1-1* mutant. The 7-8th leaves (counting from the bottom) of 4-week-old plants were treated under the condition

which was described in Fig. 3-1. Total protein was extracted and analyzed by immunoblotting using an anti-CLH1 antiserum. WT showed a signal at about 35 KD, which is consistent with the size of CLH1 protein estimated from the *CLH1* coding sequence. In contrast, no signal was obtained from the protein extracts of the *clh1-1* mutant (Fig. 3-1), indicating that the antibody is specific to CLH1. Although we attempted to detect CLH2 protein with an anti-CLH2 antiserum, we could not detect the specific signal of CLH2 in WT.

Then we analyzed the content of CLH1 during dark senescence with or without 50 μ M MeJA treatment. Consistent with the reported patterns of mRNA expression (Tsuchiya et al. 1999) (Kariola et al. 2005), the expression level of *CLH1* was slightly induced during dark-induced senescence in the absence of MeJA and it was much enhanced (more than two times) by treating with 50 μ M MeJA (Fig. 3-1) .

In vitro measured CLH activity was correlated to the CLH1 protein level (Fig. 3-2). Compared to the leaves before senescence, CLH activity was slightly increased after 2 days dark treatment without MeJA treatment. However, the WT leaves in the same condition treating with 50 μ M MeJA, CLH activity was induced more than two times (Fig. 3-2). Hence, the CLH protein level was increased by darkness and it was enhanced by MeJA treatment. Since the CLH activity and protein levels

are correlated during MeJA treatment, it is concluded that the activation of CLH is not necessary. On the other hand, in the *clh1-1* mutant which lack of CLH1, only about 10% of CLH activity were retained. In addition, MeJA treatment could not enhance the activity of CLH in this mutant. It is possible that CLH2 contributes to the activity observed in the *clh1-1* mutant. These results implied that CLH1 contributes most of the activity of CLH in Arabidopsis.

3.3.2 The *clh1-1* mutant showed a similar Chl breakdown rate with WT even during MeJA-induced senescence

Although CLH1 was absent in the *clh1-1* mutant, we could not observe any difference in the phenotype of green plants between the *clh1-1* mutant and its ecotype (Fig. 3-3). That is reasonable, because the knockout plants which absence enzymes which involved in Chl catabolism, usually no any visible difference phenotype before senescence, compared with WT (Pružinská et al. 2003) (Kusaba et al., 2007) (Schelbert et al., 2009) (Meguro et al., 2011).

It was suggested that CLH1 might be involved in Chl degradation only under some stress conditions (Azoulay Shemer et al. 2008). Because under stress conditions, Chl degradation happened much faster and CLH1 expression levels were also much enhanced. For example, MeJA promotes leaf senescence and Chl degradation (He et al. 2002), at the same time, it also enhances the expression levels of CLH1 ((Tsuchiya et al. 1999) (Kariola et al. 2005) and this study, see Fig.

3-1 and 3-2). To check the possibility of this hypothesis, we performed dark-induced leaf senescence with or without 50 μ M MeJA treatment. All leaves from 4-week-old plants of the different lines were detached and kept in dark with buffer in the absence or presence 50 μ M MeJA for 4 days. The results were shown in Figure 3-3. Without MeJA treatment, the leaves of both single and double mutants of CLHs turned yellow as fast as WT during dark incubation, whereas in the *pph* knockout mutant (It was demonstrated that PPH is involved in Chl degradation in *Arabidopsis*), all leaves remained green after 4 days of dark incubation. The average Chl content per unit area of leaves was also measured (Fig. 3-3). Decrease in Chl content was not suppressed in both of the *clh1-1* and *clh1-1/2-2* double mutants. In other words, the decrease of Chl content in the *clh1-1* and *clh1-1/2-2* mutants were similar with WT. If CLHs were involved in Chl degradation like PPH, the Chl degradation rate should be slower in *clh* mutants.

The rate of senescence and Chl degradation was strongly accelerated by MeJA treatment in WT (Fig. 3-3). However, the Chl degradation rate was also enhanced in *clh* mutants by treating with 50 μ M MeJA. Additionally, the Chl degradation rate of leaf in CLHs mutants was similar with WT. On the contrary, the leaves of PPH mutant showed stay-green phenotype and retained much more Chl than the other lines after MeJA treatment (Fig. 3-3). At the same time, we noticed that the content of Chl was retained in-dependent MeJA treatment in PPH mutant. These

results implied that the PPH pathway for Chl degradation can be promoted by MeJA treatment. In other words, MeJA promotes Chl degradation through the PPH pathway rather than CLH.

Mutants of the enzymes for Chl metabolism are known to accumulate intermediate molecules of Chl synthesis or degradation. Altering the expression of CLH1 in transgenic *Arabidopsis* caused changes in the Chl-to-Chlide ratio was reported (Benedetti and Arruda 2002). To investigate the effect of absence of CLHs on Chl metabolism, Chl and its derivatives were extracted from leaves of the WT and *clh* mutants and analyzed by HPLC. We found that the content of Chl *a* and Chl *b* were similar between the WT and CLH mutants before and after dark-induced senescence with and without 50 μ M MeJA treatment (data are not shown). And we also noticed that there was almost no Chlide detected by HPLC if we always extract pigments at sub-zero temperatures (Fig. 2-4).

3.3.3 CLH1 is located to both tonoplast and ER membrane rather than chloroplast

CLH1-eYFP (C-terminal fusion) was over-expressed in *Arabidopsis* to determine the subcellular localization of CLH1. After getting the stable CH1-eYFP over-expression lines, protoplasts were isolated from the leaves and they were observed by using a confocal microscope. From the fluorescent image, it was clear that the fluorescence of eYFP (yellow) was located outside of the chloroplast (red), and it

was most likely located outside of the chloroplast, and on some membrane structure (Fig. 3-4). This result was according to the transient expression experiment which was reported in 2007 (Schenk et al., 2007). Comparing the localization of GFP marker of ER (GFP-ER protein) and tonoplast (GFP- δ -TIP) (Fig. 3-4) (Cutler et al., 2000), it was difficult to demonstrate CLH1-eYFP was located to the ER or tonoplast, or both of them.

It was reported that when target protein was fused with GFP, the localization of this target protein would be interfered due to masking of amino- and carboxy-terminal targeting and modification signals (Tian et al., 2004). Therefore, the localization of native CLH1 would be required.

To examine the localization of native CLH1 in *Arabidopsis* leaves, we first prepared membrane and soluble fractions from WT leaves and checked the CLH1 by immunoblotting. The result showed that CLH1 was detected in the membrane fraction rather than soluble fraction (Fig. 3-5). The marker proteins, BiP2 for the ER and V-PPase for the tonoplast were mainly detected in the membrane fraction, suggesting the majority CLH1 is associated with the membrane. However, a little amount of CLH1 was detected in the soluble fraction. At the same time, we detected a little amount of ER marker protein BiP2 in the soluble fraction. This signal should be the contamination of membrane proteins into the soluble fraction.

It was reported that CLH1 was detected in vegetative vacuole by proteome analysis besides the reports of the chloroplast localization (Carter et al. 2004). In this study, both intact chloroplast and vacuole were isolated from WT and the *clh1-1* mutant, and then the samples were used to determine CLH1 by immunoblotting. In WT, CLH1 was concentrated in the vacuole sample, while we could not detect any CLH1 in the sample of chloroplasts even 3 times of the total protein amount were loaded (Fig. 3-5). This result showed that native CLH1 is located to the tonoplast rather than to the chloroplast (Fig. 3-5).

In order to confirm the tonoplast localization of CLH1 and to test whether CLH1 is also located to other kinds of membrane, we try to separate different types of membrane. Then the sucrose (Suc) density gradient centrifugation experiments were performed. Total subcellular fractions were extracted from leaves by membrane extraction buffer containing either Mg^{2+} or EDTA. The association of ribosomes with the ER is Mg^{2+} -dependent, therefore depletion of Mg^{2+} from the extraction buffer results in dissociation of ribosomes from the ER, then the ER will be redistributed to lower Suc density position, comparing with the ER which associated by ribosome after fractionation (Lord et al., 1973) (Lord, 1987). After Suc density gradient centrifugation of the WT membrane sample, and sample collection (see Materials and Methods section), the distribution of CLH1 and marker proteins was analyzed by immune-blotting. The position of the membrane-

bound fraction of the ER marker BiP2 was clearly shifted upon depletion of Mg^{2+} ions from the extraction buffer, whereas the pattern obtained for the markers of the plasma membrane (PM) and the tonoplast were almost independent of the presence or absence of Mg^{2+} ions (Fig. 3-6). Relative Chl content, which was measured as a marker for the thylakoid membrane, also did not show a Mg^{2+} dependent shift like BiP2. However, the position of CLH1 in the gradient shared the similar characteristic Mg^{2+} shift with BiP2, and it was much different from the position of plasma membrane (PM) (from 15 to 17) and thylakoid membrane (from 13 to 17) in the gradient under both absence and presence of Mg^{2+} . These results implied that CLH1 located to the ER rather than the PM and thylakoid membrane. However, we also noticed that the positions of CLH1 and BiP2 showed differences under both absence and presence of Mg^{2+} condition. On the other hand, the overlapping patterns between CLH1 and tonoplast were also observed under both conditions. In the absence of Mg^{2+} , CLH1 could be detected in fractions from 5 to 13. While BiP2 was concentrated in fraction 5 and 9 to 15, and V-PPase was concentrated in fractions from 9 to 15. In the presence of Mg^{2+} , CLH1-containing fractions were detected mainly in fractions 11 to 17. Under the same condition, BiP2 was concentrated in fractions from 11-19 and V-PPase was concentrated in the fractions of 9-17 (Fig. 3-6). Taken together, the results indicated that both the tonoplast and ER associated with CLH1.

It was reported that citrus CLH was imported into the chloroplast during ethylene-induced Chl breakdown (Azoulay Shemer et al., 2008). So we also check whether CLH1 could import into chloroplasts after phytohormone (MeJA) treatment. We performed membrane fractionation on Suc density gradient centrifugation without Mg^{2+} after the leaves were incubated with 50 μM MeJA added buffer for 3 days. The immuno-blotting results showed that the positions of CLH1 and markers in the gradient were very similar with the results using leaf sample which before senescence (Fig. 3-6, 3-7). Most of the CLH1 was not detected at the similar patterns of relative high Chl content (thylakoid marker). However, the position of CLH1 in the gradient was also similar with BiP2 and V-PPase. Taken together, we concluded CLH1 is located to both the ER and tonoplast with or without MeJA treatment in Arabidopsis leaves.

3.3.4 Chlide *a* and Pheide *a* delayed the development of *Spodoptera litura* larvae, and also reduced the survival rates.

To demonstrate whether Chlide and Pheide are potential poison to insect herbivores, We check the poisonousness of Chlide *a* and Pheide *a* by comparing with Chl *a*. After the larvae of *Spodoptera litura* were hatched, the larvae of similar sizes were selected and were fed with artificial food that was mixed with the same concentration of N, N'-dimethylformamide (DMF) but different kinds and concentrations of pigments (see Materials and methods section). After feeding

for 11 days, the growth and development status of each larva was surveyed (Fig. 3-8). The result showed that in the control and Chl *a* 400 nmol/g groups, the development of larvae were similar with each other. More than 80% of the larvae which were fed without pigments or 400 nmol/g Chl *a* added food developed to the 4th instar. However, only 56% and 63% of larvae developed to 4th instar which were fed with Chlide *a* 400 nmol/g and Pheide *a* 400 nmol/g added food respectively. About 61% larvae developed to 4th instar after they were fed with 200 nmol/g Chlide *a*. The statistic analysis showed that the development rate was significantly delayed by feeding with 400 nmol/g Chlide *a*, however, it was not delayed by feeding with the same concentration of Chl *a*. We also noticed that the larvae who were fed with Chlide *a* or Pheide *a* also showed higher death rates than those fed with Chl *a*. The results showed Chlide *a* and Pheide *a* delay larvae development and even kill a population of larvae. These results indicate that possibility of plants use the two Chl-derivatives to defense against insect herbivores.

3.3.5 Chlide *a* was formed after leaf cells were disrupted by homogenization

We have demonstrated that CLH1 was located outside of chloroplasts, while Chl was inside of chloroplasts. Moreover, there was almost no Chlide existed in leaves (see Chapter 2). We expected that only after leaf cells were disrupted by insect herbivores in larvae's mouth and gut, Chlide and Pheide would be produced. In the

midgut of insect larvae, the variation of pH values exists among different species. The midgut of some insect larvae including lepidopteran species is alkaline (Dow 1992). Therefore, to mimic the leaves after they have been eaten and disrupted in the mouth and midgut of different insect species, *Arabidopsis* leaves were homogenized at pH8 or pH10 (Fig. 3-9). After homogenization, Chlide was detected in all of the plant lines which we have tested. Under the same condition, Chlide *a*/Chl *a* ratio in WT was much higher than in the *clh1-1* mutant, but lower than in the CLH1-YFP overexpression lines. Comparing with pH8 conditions, about 1 times more Chlide was formed in pH10 conditions. After leaves were homogenized and incubated at room temperature for 0.5 h, the Chlide *a*/Chl *a* ratio in WT was 0.08 and 0.2 under pH8 and pH10 conditions respectively, While in C1Y-15 lines, the Chlide *a*/Chl *a* ratio was as high as 0.2 and 0.5 respectively (Fig. 3-9). Comparing with 0.5 h, we found the Chlide *a*/Chl *a* ratio was just slightly increased when incubation was performed at the same condition for 2 h. The results suggested that CLH activity was high and the reaction takes place in the early stage after leaf cells were just disrupted.

3.3.6 CLH is involved in plant defense against insect herbivores

In order to demonstrate whether CLH was really involved in plant defense against insect herbivores, plants feeding experiments were subsequently performed. After feeding the young larvae (just after hatching) of *Spodoptera litura* for 11 days,

almost all of the larvae who fed with the WT and *clh1-1* mutant were survived. Statistic analysis showed that there was no significant difference between the two larvae groups. Comparing with the larvae fed with the WT and *clh1-1* mutant, the average survival rates of larvae who ate the three CLH1-YFP overexpression lines were significantly decreased under 0.05 significant levels (Fig. 3-10). These results indicated that CLH1 was involved in plant defense against insect herbivores.

3.4 Discussion

In green leaves, an enzyme that catalyzes the hydrolysis of Chl to phytol and Chlide was discovered about 100 years ago (Stoll, 1912) (Willstätter and Stoll 1913). It was named as CLH. After that, many studies have been done on CLH, and it has been widely considered to exist in higher plants and algal (Jeffrey and Hallegraeff, 1987) (Shioi et al. 1991). For a long time, scientists proposed and believed that CLH was the first enzyme that involved in Chl degradation pathway (Hörtensteiner, 2006). Along with the development of modern molecular genetics, it was possible to analyze the function of CLH in vivo, by using the information obtained from the CLH gene sequence and the *clh* mutants which lack CLH proteins. One of the Arabidopsis CLH isoforms, CLH1 was first named as ATHCOR1, because it was cloned as a gene in response to coronatine, MeJA and wounding in Arabidopsis (Benedetti et al., 1998). It was found that it is expressed

in seedlings, young and mature leaves, flowers and siliques, but its expression is not detected in roots. In the next year, Tsuchiya and coworkers cloned two homologous genes from *Arabidopsis* those encode two enzymes (CLH1 and CLH2) which possessed CLH activity in vitro (Tsuchiya et al., 1999). CLH1 mRNA was induced rapidly by a treatment of MeJA, and a high mRNA level was maintained up to 9 h. CLH2 was constitutively expressed at a low level in the rosette leaves and flowers, and did not respond to MeJA. Interestingly, no typical feature of a signal sequence for chloroplast was identified in CLH1, and also CLH2 has only a weakly predicted signal sequence for chloroplasts (Tsuchiya et al., 1999). In addition, both CLH1 and CLH2 fused with GFP was located in cytosol when they were transiently expressed in *Arabidopsis* protoplast (Schenk et al., 2007). Based on these findings, two hypotheses were proposed. Firstly, another Chl degradation pathway in which CLH1 is involved exists outside of chloroplasts in plants (Takamiya et al., 2000). Another hypothesis was that CLH1 can be imported into chloroplasts and degrade Chl after some stress treatment. The second was constructed based on the research on citrus CLH (Azoulay-Shemer et al., 2008). However, through studying the CLH1 and CLH2 knockout single and double mutants, no obvious difference was observed between these mutants and WT during natural and dark-induced senescence and Chl breakdown in *Arabidopsis*

(Schenk et al., 2007). Our results were in accordance with this report when leaf senescence and Chl degradation was induced in darkness.

However, it was reported that citrus CLH functions as a rate-limiting enzyme in Chl catabolism during ethylene-induced fruit color-break (Harpaz-Saad et al., 2007) (Azoulay Shemer et al. 2008). For CLH1 in Arabidopsis, the expression of the *chl1* gene was daftly induced by MeJA treatment (Tsuchiya et al., 1999). Because MeJA is also a well-known phytohormone that promotes leaf senescence (He et al., 2002), it is suggested that Chl was degraded by CLH1 during MeJA-induced senescence (Azoulay Shemer et al., 2008). Our results showed that after treating leaves with 50 μ M MeJA for 4 days in darkness, Chl degradation rate was strongly accelerated by treating with MeJA in both WT and the CLH1 knockout mutant. Although both CLH protein and activity were enhanced by MeJA treatment (Fig. 3-1 and 3-2), the Chl degradation rate was also similar between the same age of leaves of *chl1-1* (CLH1 deficient mutant) and WT. In addition, the *CLH1* and *CLH2* double mutant also degrade Chl at the similar rate with WT after MeJA treatment (Fig. 3-3). These results suggested that the expression level of CLH1 was not related to the Chl degradation rate. Absence of either or both of CLH1 and CLH2 had no obvious effect on Chl metabolism in Arabidopsis. During the experiment, we noticed that similar content of Chl was retained in the *pph* mutant under the absence and presence of 50 μ M MeJA. The results implied that MeJA

treatment promoted Chl degradation through the PPH degradation pathway rather than CLH pathway. Taken together, we would like to suggest that CLH1 is not involved in Chl degradation even in the presence of MeJA during leaf senescence.

The intracellular localization of CLH has also been studied for a long time (Krossing, 1940) (Ardao and Vennesland, 1960) (Terpstra, 1974) (Tarasenko et al., 1986) (Matile et al., 1997). There were certain discrepancies concerning the localization of CLH among these reports, a consensus seemed to be its localization in the chloroplasts. It is reasonable to assume that CLH is present inside of the chloroplast if it is involved in the first step of Chl degradation, because its substrate, Chl is degraded inside of the chloroplast till it becomes colorless products (Hörtensteiner, 2006). The intermediate pigments during degradation, such as Chlide, Pheide and the enzyme PAO are all located in the chloroplast (Hörtensteiner, 2006). In addition, CLHs which were fused with GFP both at the C-terminal and N-terminal were located in the cytosol when they were transiently expressed in the protoplasts of *Arabidopsis* (Schenk et al., 2007). It was suggested that GFP fusions may interfere with the natural localization of CLHs due to masking of amino- and carboxy-terminal targeting and modification signals (Tian et al., 2004) (Azoulay Shemer et al., 2008). Therefore, to demonstrate the localization of CLH1, it was necessary to check the localization of natural CLH1 without any modification.

CLH has been considered as a membrane protein (Lambers et al., 1984) (Matile et al., 1997), but no potential membrane spanning region was found in any of the CLHs cloned. It was unclear whether natural CLHs are a soluble protein or membrane associated protein or whether both forms exist in plants (Ogura, 1972) (Kuroki et al., 1981). Firstly, we detected CLH1 in the membrane fraction (Fig. 3-5). So we would like to conclude that CLH1 is a membrane associated protein. Furthermore, there was another publication showing that CLH1 was detected in vacuole samples by proteomic analysis (Carter et al., 2004). To confirm that CLH1 was located to the tonoplast, intact vacuole and chloroplast were both isolated from WT and *chl1-1* respectively. The results showed that CLH1 was concentrated in the vacuole sample and no CLH1 signal was observed in the chloroplast sample. The result clearly implied that CLH1 was located to the tonoplast rather than chloroplasts. In this work, the results of membrane fractionation with and without Mg^{2+} told us that CLH1 also located to ER besides the tonoplast. In diatom *P. Tricornutum*, CLH was found as a glycoprotein (Terpstra, 1981) (Terpstra et al., 1986), it was also assumed that native *Ca*CLH was modified by glycosylation at the Asn residue, suggesting a modification (glycosylation) in ER, because ER is a major site for the glycosylation of proteins. For *Ca*CLH, Tsuchiya and coworker tried many times to isolate the gene encoding chloroplast-type CLH by the heterologous screening of a *C. album* cDNA library, but they failed (Tsuchiya et

al., 1999). In addition, *CaCLH* possesses a signal sequence at the N terminus for ER (Tsuchiya et al., 1999), however, there was no signal sequence for ER retention and 11 aa residues existed between the putative cleavage site in ER and the N terminus of the putative mature *CaCLH*, so it was assumed that *CaCLH* was transported to other organelles, for example vacuole. Taking into account this observation and the results of our study (Fig. 3-5, 3-6), we conclude that CLH1 in *Arabidopsis* cell was located to both the tonoplast and ER. We also performed membrane fractionation using leaves which were kept in darkness and treated with MeJA for three days. The results showed that the intracellular localization of CLH1 was not changed after MeJA treatment. So we conclude that CLH1 is not transported into chloroplast after MeJA treatment. That was much different from the citrus CLH, which is located in the chloroplast in ethylene-induced fruit color-break (Azoulay Shemer et al., 2008).

On the other hand, It was also suggested that Chl is transported outside of chloroplast and it is subsequently degraded by CLH1 in *Arabidopsis* (Takamiya et al., 2000). One kind of protein, water-soluble Chl protein (WSCP) are assumed to act as Chl carrier proteins transporting Chls from thylakoid membranes to the active site of CLH to initiate the process of Chl degradation (Matile et al., 1997). However, WSCP is only exists in several plant groups, and the Chl-binding function of WSCP was just demonstrated in vitro (Kamimura et al. 1997) (Satoh et

al. 1998) (Takahashi et al. 2012). However, CLH exists in plant and algal kingdom universally (Stoll, 1912) (Jeffrey and Hallegraeff, 1987) (Shioi et al. 1991). On the other hand, Chl was degraded inside of chloroplast before it was degraded to a colorless intermediate (Hörtensteiner, 2006), except in a few cases that some Chl or its green derivatives was detected in vacuoles of tobacco and *Arabidopsis* (Martíne et al., 2008) (Wada et al., 2008). In tobacco, Chl *a* was detected in senescence-associated vacuoles (SAVs) at a relatively slow rate but not in leaves treated with ethephon. In *Arabidopsis*, small chloroplast without stroma was detected inside of vacuole when autophagy was happening (Wada et al., 2008). It was reported that autophagy happens in dark-induced senescence leaves (Wada et al., 2008), but we could not detect any obvious difference about the Chl degradation rate between CLH mutants and WT during dark-induced senescence (Fig. 3-3). Taken together, we suggested that even some of Chl was degraded in the vacuole when autophagy happening, CLH1 also not involved in Chl degradation. Because small chloroplasts were located inside of vacuole when autophagy happened (Wada et al., 2008), while CLH1 was associated to tonoplast and ER membrane. Chl SAVs may be degraded by other enzymes or non-enzymatic tautomerization in acidic vacuole.

It was reported that citrus CLH is a rate-limiting enzyme in Chl catabolism and it was located to the chloroplast in ethylene-induced fruit (Azoulay Shemer et al.

2008), the function of CLH may be different among different plant systems (Amir-Shapira et al., 1987) (Azoulay-Shemer et al., 2011). Combining the intracellular localization of CLH1 with the phenotype of *clh1* and *CLHs* double knockout lines during dark-induced senescence with or without MeJA, we conclude that CLH1 is not involved in Chl breakdown during MeJA-induced senescence in Arabidopsis. What is the true function of CLH1 in Arabidopsis? It was reported that CLH1 may play a role in the response to pathogens and wounding in Arabidopsis (Kariola et al., 2005). We suggest one CLH work model: when the leaves are infected by pathogens or bitten by herbivores, plant cell structure will be disrupted. CLH subsequently may have a chance to react with Chl to produce Chlide and its catabolites. In fact, when leaf cells were disrupted, much Childe was formed easily both under pH8 and pH10 conditions.

In higher plants, Chl degradation is an important catabolic process of leaf senescence and fruit ripening. In addition, it also occurs as a response to many biotic and abiotic stresses (Hörtensteiner and Kräutler 2011). Therefore, Chl degradation has been studied for many years. With the key enzymes identified, the degradation pathway was almost clarified (Tsuchiya et al. 1999) (Pružinská et al. 2003) (Pružinská et al. 2007) (Schelbert et al. 2009) (Hörtensteiner and Kräutler 2011). On the other hand, some researchers found some of the Chl degradation intermediates have some special physiological functions for plants and other

organisms. For example, in *Arabidopsis*, light-independent cell death can be induced by accumulation of Pheide *a* (Hirashima et al. 2009). In addition, Pheide *a* possess cytotoxic activity to cancer cells (Sowemimo et al. 2012). Then we compared the hydrophilicity of Pheide *a* with Chl *a*. By removing the phytol side chain and Mg^{2+} from Chl *a*, Pheide *a* becomes to a hydrophilic compound, while Chl *a* is a hydrophobic compound. Because Mg^{2+} cannot affect the hydrophilic ability of the pigments, so Chlide is also a hydrophilic compound. The results of pigments feeding experiments implied Chlide *a* and Pheide *a* were poisonous, while Chl *a* was not. We expected that plants use Chl for photosynthesis, on the other hand, the Chl-derivatives can be used to defense against insect herbivores.

To minimize the risk of self-intoxication, defense compounds are often stored in the compartments of limited metabolic activity (Mithöfer and Boland 2012). One of the famous examples is hydrogen cyanide (HCN), which is released from cyanogenic glycosides. Cyanogenic glycosides are not toxic and are stored in the vacuole, whereas the enzyme - glycosidase is present in the cytoplasm. Only after the cells were disrupted by the consumption of insect herbivore, the enzyme and the substrate get chance to react with each other. Subsequently, acetone cyanohydrin is released, which can be converted into HCN and acetone either spontaneously or by a hydroxynitrilelyase (Vetter 2000). We expected that the plants may use Chlide and Pheide to defense against insect herbivores through

CLH activity after leaves were consumed by insects. After homogenization experiments were performed to mimic the leaf cells were disrupted by insect eating and digestion. Chlide was formed and the amount was positively correlated with the CLH1 expression level. In addition, the CLH1 overexpression lines were more poisonous to the larvae of *Spodoptera litura* (Fig. 3-10). While almost all the larvae were survived after feeding WT and the *clh1-1* mutant. Statistic analysis showed that there was no significant difference between the two larvae groups. However, plants show variation in the amount of CLH (Bacon and Holden 1967) (Shioi et al. 1991). CLH involved defense system maybe not important for *Arabidopsis*, however, it should be one of the important defense system for the plants which have the capacity to induce more CLH after insect attack happen such as *Glebionis coronaria* (garland chrysanthemum) (Shioi et al. 1991). On the other hand, different insects and different stages of insects will show different tolerance level to the poisonousness of Chlide and Pheide. It is possible some insects more sensitive to this plant defense system than *Spodoptera litura* which was employed in this study. Lastly, plants utilize Chl-derivatives as chemical defense compounds through CLH activity possibly more effectively to chewing insects because large amount of leaf cells were broken during chewing and digestion. While piercing-sucking insects, such as aphid, they usually take the tissue juice by piercing and sucking without many leaf cells disruption, therefore,

only little amount of Chlide and Pheide will produced in the body of such kind of insect.

Comparing the results of pigment feeding and plant feeding experiments, we noticed that although more larvae were killed by feeding Chlide or Pheide, and also by feeding CLH1-overexpression leaves, the significant delaying of development only observed in the pigments feeding experiment (Fig. 3-8 and Fig. 3-10). In his study, the growth conditions of the pigments feeding experiment were controlled very similar for each larva. While in the leaf feeding experiment, because plants contain many defense systems, other defense systems will interfere the function of Chl-derivatives system. Except some larvae were killed directly by all the defense systems, the development of each larva varied obviously dependent on their micro-environment. Therefore, no obvious difference on development among the larvae in different groups which were fed with different plant lines.

Using Chl-derivatives as chemical defense compounds are quite convenient and low cost. Firstly, the defense mechanism is safe for the plants themselves (some defense compounds are also toxic to the plants themselves), before consumption by the insects, no Chlide and Pheide are formed. Secondly, the substrate Chl is universally and abundantly exists in the plant kingdom. Without insect attack, Chl is the major pigments for photosynthesis. On the other hand, CLH also exists in

almost all of the plant species (Drazkiewicz and Krupa 1991) (MCM Chen et al. 2012). Therefore, this defense system should widely exist in the plant kingdom. Thirdly, unlike nicotine and some other defense compounds, long pathway biosynthesis and long distance transportation is not necessary for defense against insect herbivores by using Chl-derivatives as defense compounds. This is the first report about plants employing Chl-derivatives as defense compounds against chewing insect herbivores. In future, the mechanism of action of these Chl-derivatives to insect larvae, the importance of this defense system for different plants and the sensitivity of different insects to this system should be clarified.

3.5 FIGURES, TABLE AND LEGENDS

Table 3-1 Gradient profiles for separating Chlide *a* and Chl *a*

	Time (min)	Solvent A Methanol: acetonitrile: aqueous pyridine (50:25:25 v: v: v) %A	Solvent B Methanol: acetonitrile: acetone (20:60:20 v:v:v) %B
Chlide <i>a</i>	0 min	60	40
	11 min	0	100
	25 min	0	100
	27 min	60	40
	30 min	60	40
Chl <i>a</i>	0 min	0	100
	50 min	0	100

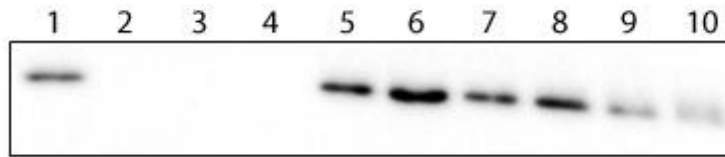


Figure 3-1

Figure 3-1. The change of CLH content during senescence with or without 50 μ M MeJA. Lane 1, wild type (col) before senescence; lane 2, clh1-1 before senescence; lane 3 and 4, clh1-1; lane 5 and 6, col dark induced senescence without and with 50 μ M respectively for 2 days; lane 7 and 8, col dark induced senescence without and with 50 μ M respectively for 4 days; lane 9 and 10, col dark induced senescence without and with 50 μ M respectively for 6 days.

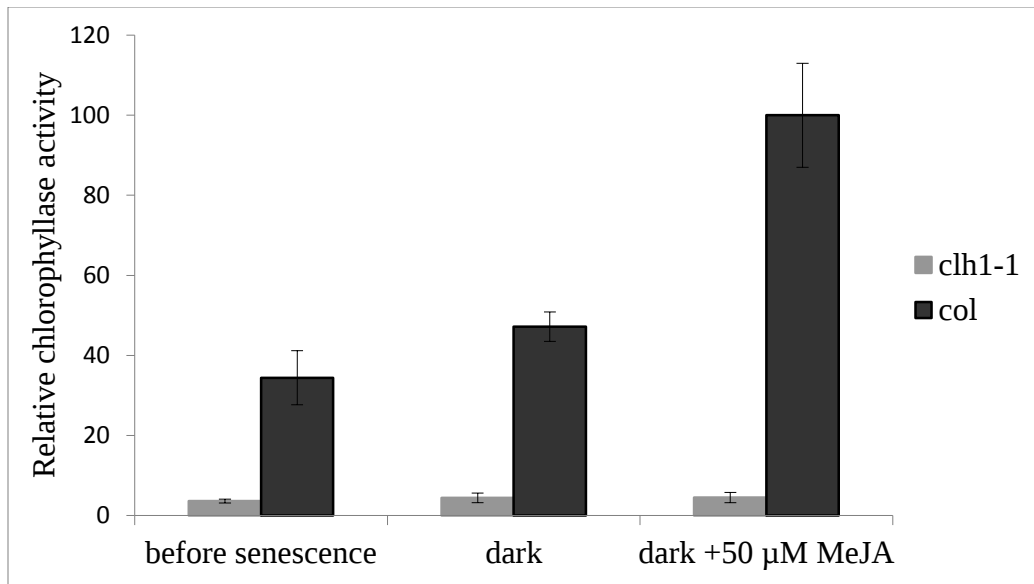


Figure 3-2

Figure 3-2. Determination of relative activity of CLH in vitro before and after senescence with or without 50 μ M MeJA. All the leaves were 7~8th leaves (counted from the bottom of the plant), and before or after 2 days senescence. CLH activity in 50 μ M MeJA treated leaves was employed as the 100% standard. Error bars indicate standard deviations. Sample size, $n = 3$.

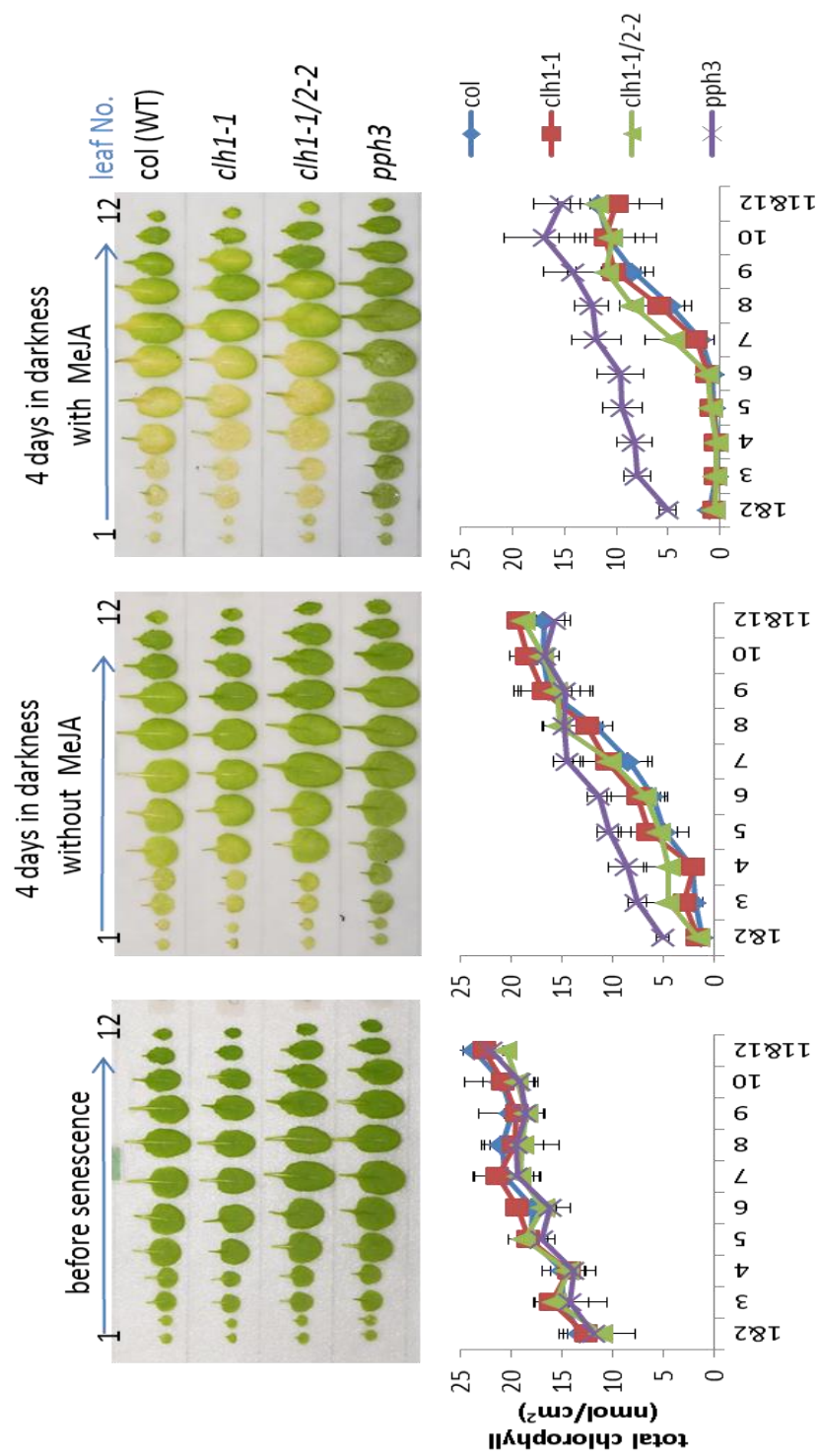
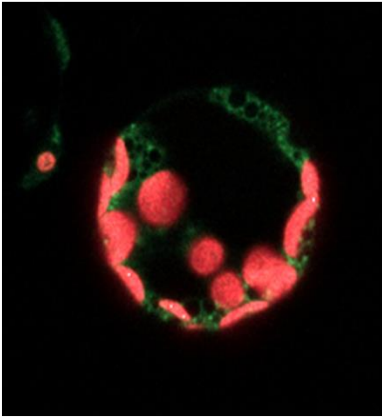


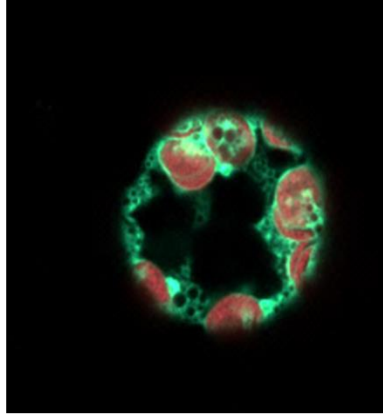
Figure 3-3

Figure 3-3. The effect of MeJA on the senescence of Arabidopsis leaves.

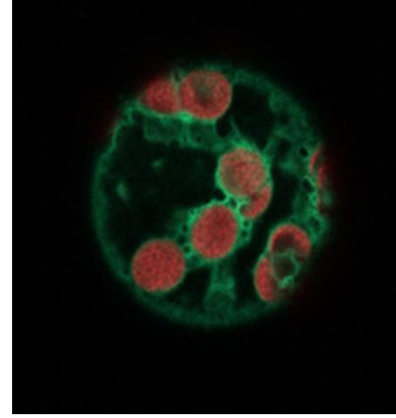
Leaves from each plant were detached and kept in darkness for 4 d in the absence or presence of 50 μ M MeJA. A. Images of the leaves during senescence. B. Total chlorophyll content in cm^2 of leaf. The leaf numbers were counted from the bottom of the 4-week-old plant. Error bars indicate standard deviations. Sample size, $n = 3$.



CLH1-YFP



GFP-ER protein
(ER marker)



GFP- δ -TIP
(tonoplast marker)

Figure 3-4

Figure 3-4. The CLH1-YFP(yellow fluorescent protein) image of isolated protoplast of 4-week-old transgenic plants. GFP-ER protein is a green fluorescent protein (GFP)-tagged endoplasmic reticulum (ER) marker line; And GFP- δ -TIP is a GFP-tagged vacuole membrane (tonoplast) marker line. Red color is the fluorescent of chlorophyll.

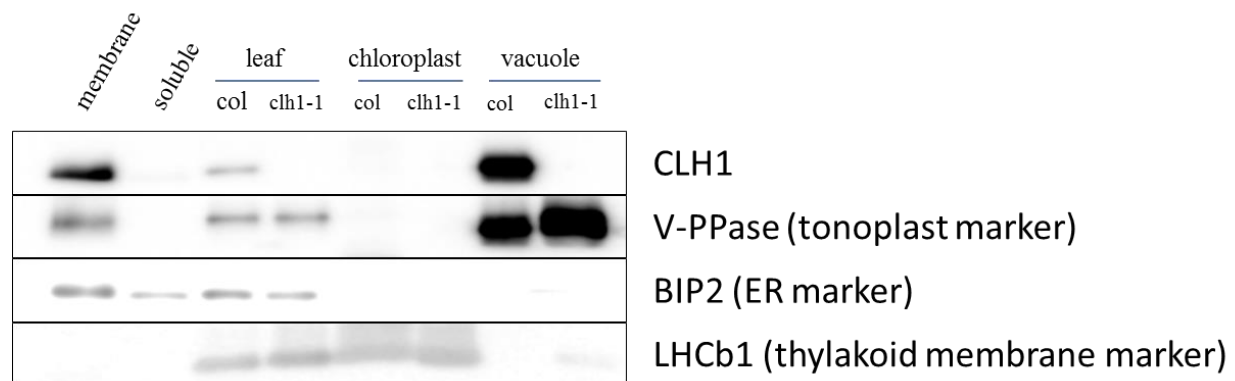


Figure 3-5

Figure 3-5. Subcellular localization of CLH1 analyzed by separation of membrane and soluble fraction and isolation of organelles. Membrane and soluble fraction were loaded with 2 µg total protein; leaf and chloroplast were loaded with 12 µg total protein; while vacuole samples were loaded with 4 µg total protein. membrane: membrane fraction; soluble: soluble protein enrich fraction.

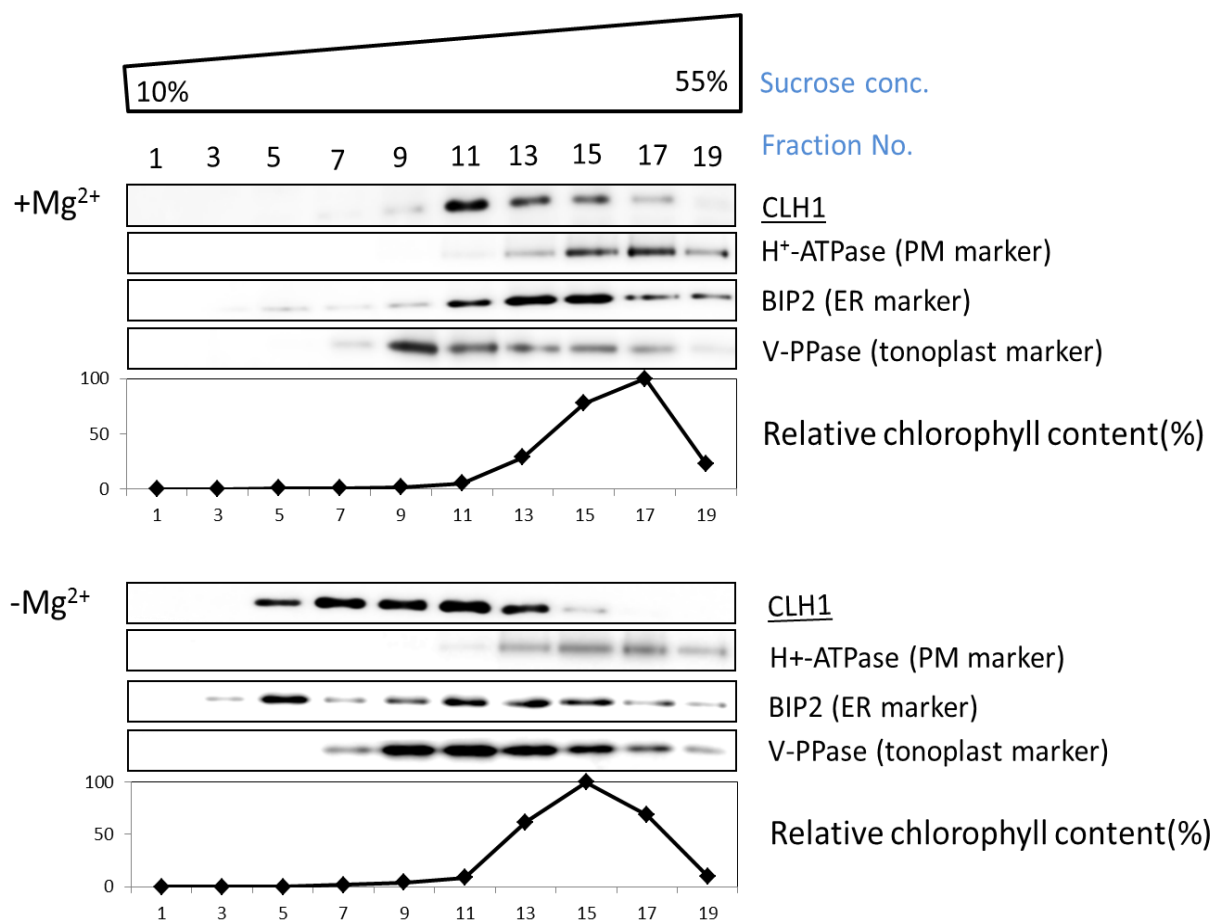


Figure 3-6

Figure 3-6. Membrane fractionation showing co-fractionation of CLH1 with both the tonoplast and ER. Sucrose density gradient centrifugation of microsome fraction with or without Mg^{2+} . After centrifugation, resulting gradient was separated into 20 fractions and then the fractions with odd number were analyzed by immunoblotting for CLH1, H^{+} -ATPase (plasma membrane (PM) marker), BIP2 (ER marker), V-PPase (tonoplast marker). Thylakoid membranes were identified by HPLC based on relative chlorophyll content(%).

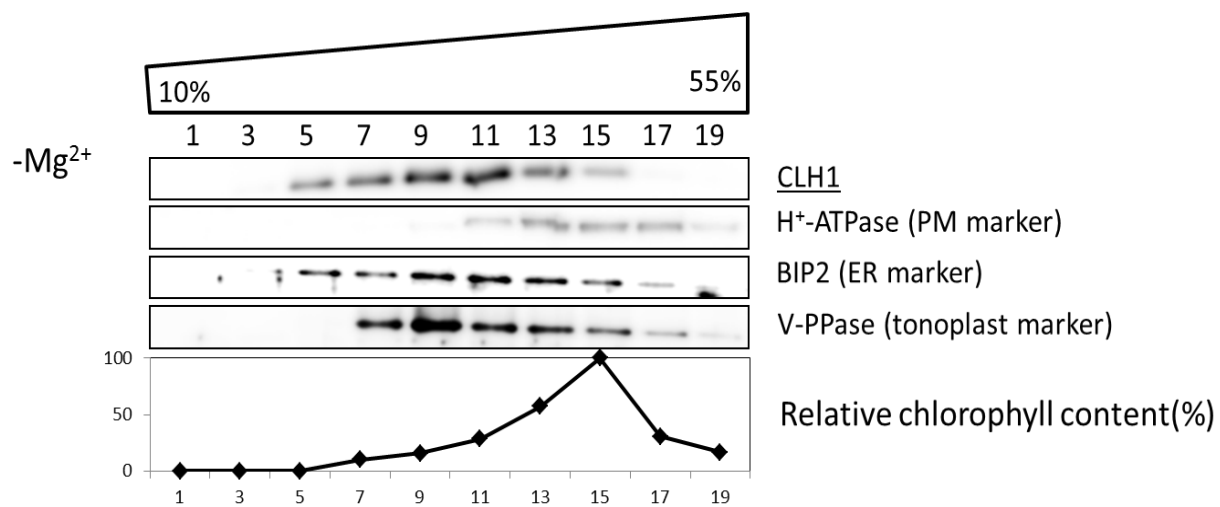


Figure 3-7

Figure 3-7. Localization of CLH1 in fractionated membranes from leaves in which senescence was induced under dark plus 50 μ M MeJA treatment for 3 days. After sucrose density gradient centrifugation of microsome fraction without Mg^{2+} , resulting gradient was separated into 20 fractions and then the odd number fractions were analyzed by immunoblotting for CLH1, H^{+} -ATPase (PM marker), BIP2 (ER marker), V-PPase (tonoplast marker). Thylakoid membranes were identified by HPLC based on relative chlorophyll content(%).

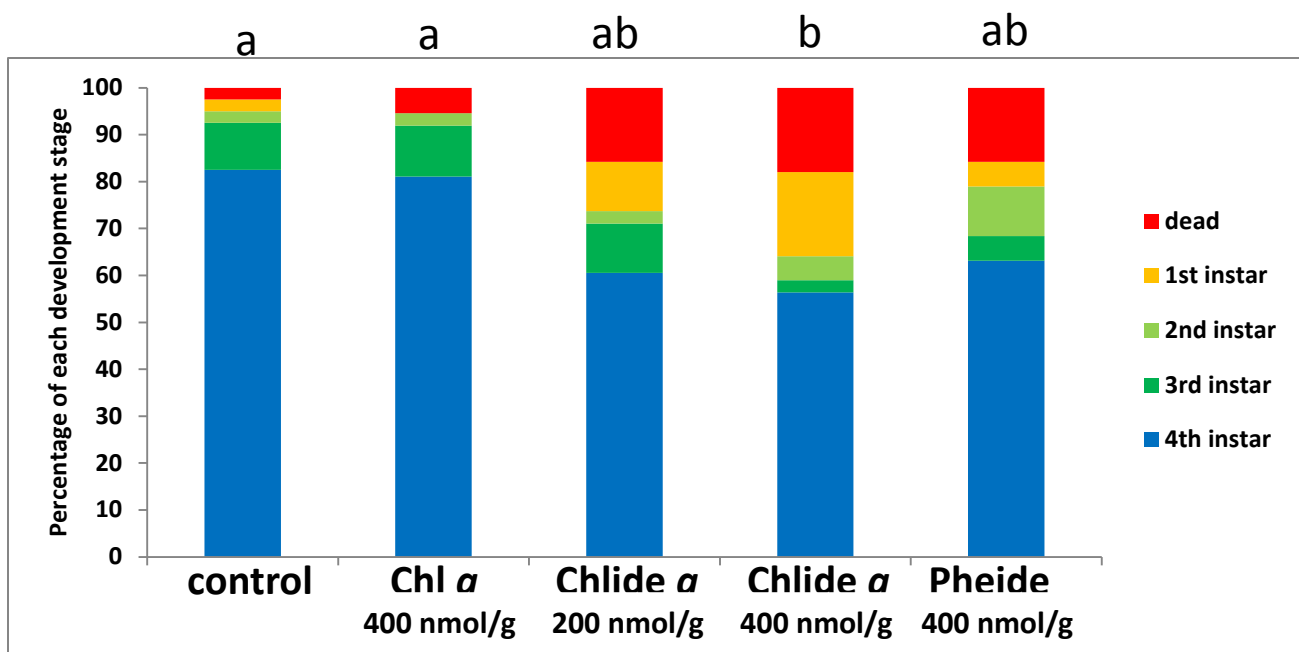


Figure 3-8

Figure 3-8. Chlide *a* and Pheide *a* delayed the development of the larvae of *Spodoptera litura*. More than 35 larvae were feeding in each group, each larva was separated and fed by the same amount of artificial food which was mixed with different pigments for 11 days. DMF was employed to dissolve pigments and the final concentration in the food was 0.1%. Chl *a*. chlorophyll *a*, Chlide *a*. chlorophyllide *a*, pheide *a*. pheophorbide *a*. The Kruskal.test and R version 3.0.1 software were employed for data statistic analysis, a and b denote $p < 0.05$.

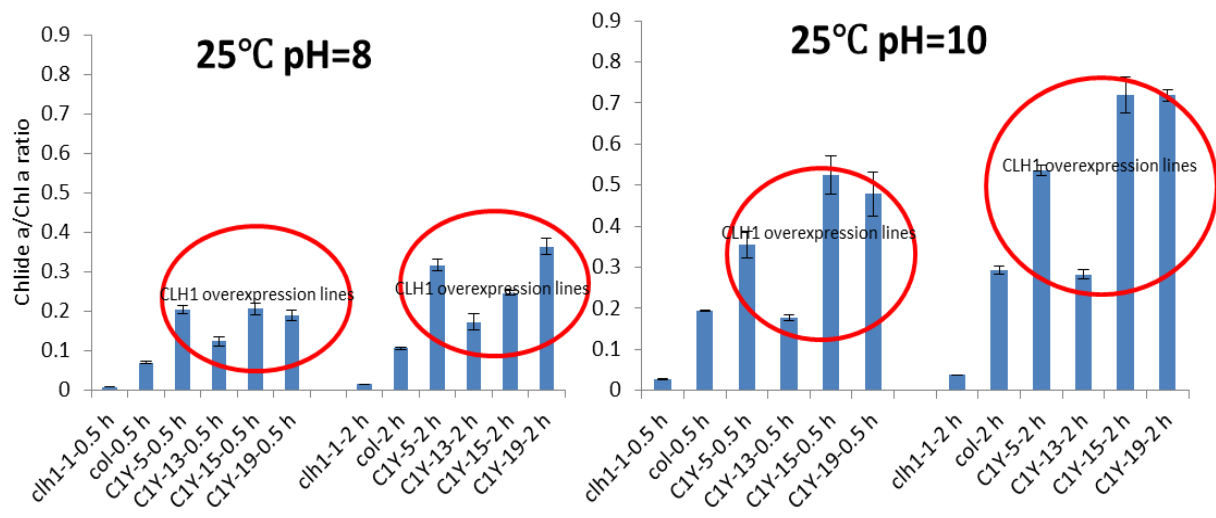


Figure 3-9

Figure 3-9 Chlide formation after leaves were homogenized in pH8 and pH10 buffer respectively. The leaves were homogenized by following the procedure which was described in the Materials and Methods section. 0.5 h and 2 h represent the period during which samples were incubated at 25°C after they were homogenized. C1Y: CLH1-YFP (*clh1-1* genetic background). In these lines, CLH1 was overexpressed by the driving of 35S promoter. Error bars indicate standard deviations. Sample size, $n = 3$.

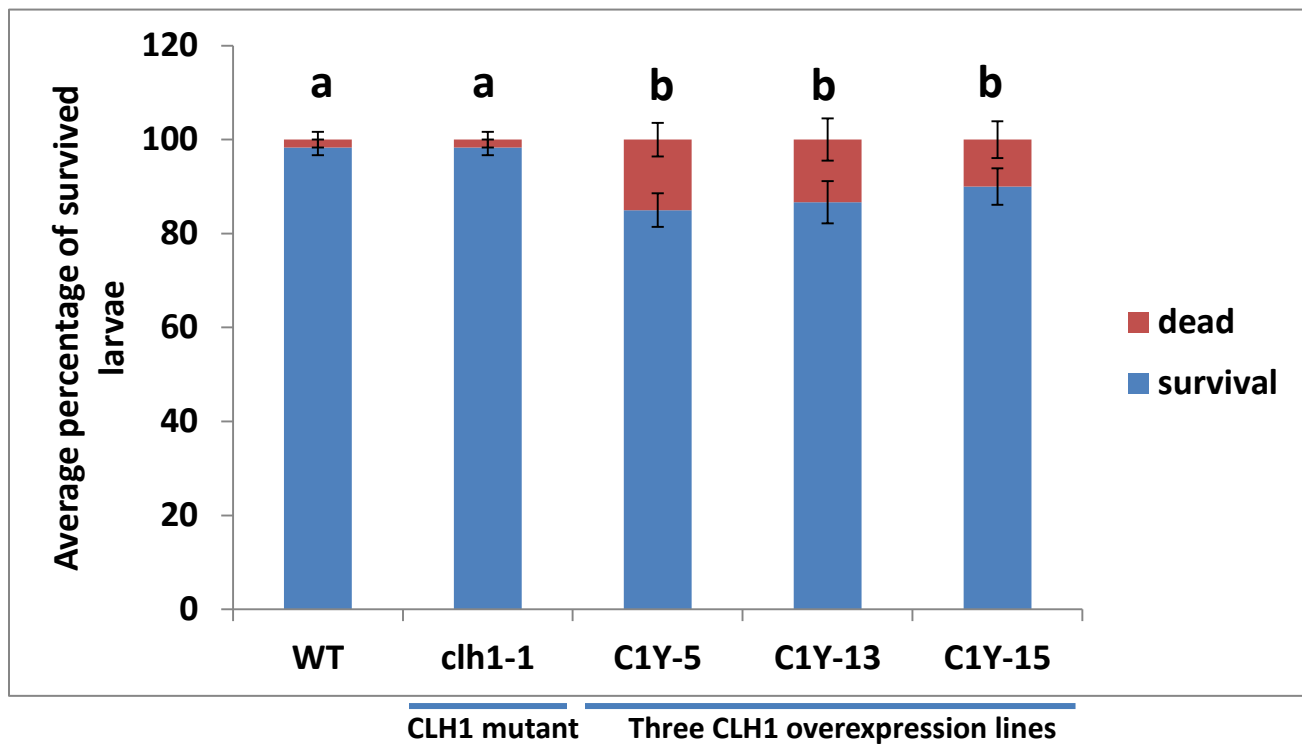


Figure 3-10

Figure 3-10 Average percentage of survived larvae by feeding Arabidopsis lines. Each plant line including 12 plants, and each plant was eaten by 5 insects. Insects were fed for 11 days. The student t-test was employed for data statistic analysis. A and B denote $p < 0.01$, a and b denote $p < 0.05$.

CHAPTER 4: CONCLUSION

In this dissertation, in order to demonstrate whether plants use Chl-derivatives to defense against insect herbivores, two Chl-derivatives which were named as Chlide and Pheide was selected as the candidates because of their hydrophilic property that is different from Chl, which shows hydrophobic characteristic. However, both in normal and stressed leaves, almost no Chlide was detected.

By the way, we noticed that Chlide is easily formed during Chl extraction at normal temperatures, even at 4°C condition. Then we demonstrated the formation of artifactual Chlide was because of the activity of the enzyme which was named as CLH. To suppress the activity of CLH during Chl extraction, and also analysis Chl more accurate by HPLC, we developed three simple methods to extract Chl. The boiling method would be a practical choice when leaves are not too thick. However, it may convert a small fraction of Chl *a* into pheophytin *a*. Extraction at sub-zero temperatures is suitable for all plant species examined in this study but may be tedious for a large number of samples and it needs liquid nitrogen and equipment for grinding leaves. Using DMF as an extractant is simple and suitable with *Arabidopsis* samples. However, this solvent cannot completely block the formation of Chlide in thicker leaves such as pea leaves.

Chlide and phytol side chains are the products of the CLH reaction on Chl. However, there was no Chlide exists in leaves. We started to doubt the function of

CLH which was considered to be involved in the first step of chlorophyll degradation. Besides CLHs were not essential for the dark induced Chl breakdown (Schenk et al. 2007). We demonstrated that the major isoform of CLH in Arabidopsis (CLH1) was not involved in Chl breakdown with MeJA treatment, even the expression level of it was much induced. On the other hand, both CLH1-YFP (C-terminal fusion) and the native CLH1 was located outside of the chloroplast. Then we hypothesized that Chlide and Pheide were used for defense against insect herbivores only after the leaves were consumed by the herbivores. We performed leaf homogenization to mimic the conditions where the leaf cells were disrupted by insect bites. When leaf cells were disrupted, much Chlide was formed easily both under pH8 and pH10 conditions. The results of pigments feeding experiments implied Chlide *a* and Pheide *a* were poisonous to insect larvae, while Chl *a* was not. In addition, the Arabidopsis in which CLH1 was overexpressed showed more poisonous to the larvae. Finally, we would like to make the conclusion that plants use Chlide and Pheide for defense against insect herbivores through CLH activity, only after plant leaves were eaten by insect herbivores. The CLH involved defense system maybe not important for Arabidopsis, however, it should be one of the important defense system for the plants which possess high activity of CLH.

Using Chl-derivatives as chemical defense compounds are quite convenient and low cost. Firstly, the defense mechanism is safe to the plants themselves. Secondly, the substrate Chl is universally and abundantly exists in the plant kingdom. The enzyme CLH also exists in almost all of the plant species (Drazkiewicz and Krupa 1991) (MCM Chen et al. 2012). Without insect attack, Chl is the major pigments for photosynthesis. Thirdly, unlikely nicotine and some other defense compounds, long pathway biosynthesis and long distance transportation is not necessary for defense against insect herbivores by using Chl-derivatives as defense compounds. In this study, we identified a new class of defense compounds. The affection of them for different class or different stage of chewing insects, and the mechanism of toxicity of them to insects are open for future research.

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