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Master thesis

阿拉伯芥葉綠素去植醇酶群之功能分析

Functional analysis of chlorophyll dephytylases in *Arabidopsis thaliana*

陳姿廷

Tzy-Ting Chen

指導教授：常怡雍 博士

Advisor: Yee-Yung Charng, Ph.D.

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摘要

葉綠素為光合作用之基本元素，參與將光能量轉化為生物能的生化反應，以滋養地球上的大多數生命。葉綠素（chlorophyll, Chl）在結構上包含了親水性的葉綠酯（chlorophyllide）與疏水性的植醇（phytol），並可藉由去植醇反應（dephytylation）將這兩部分水解開來。葉綠素去植醇反應一直被認為是植物的基礎代謝步驟，參與在不同的葉綠素相關代謝途徑，包括葉綠素之轉換、降解、構型循環及生育酚（tocopherol）生合成等，但其中執行去植醇反應的酶大多未能被確認。最近，一個新穎的葉綠素去植醇酶（CLD1）被發現可能參與葉綠素轉換時的回收再利用。蛋白序列親源分析顯示，CLD1 和參與葉片老化時葉綠素降解的脫鎂葉綠素去植醇酶（pheophytinase, PPH）以及另外兩個功能未知的蛋白具有同源性。因為後兩者的重組蛋白均有葉綠素去植醇酶活性，故被命名為 CLD2 和 CLD3。為了解這些去植醇酶的生理作用，我們建立了阿拉伯芥 *cld1 cld2 cld3*（TK）和 *cld1 cld2 cld3 pph*（QK）基因剔除突變株。正常與逆境（強光、或光度變動、或高溫、或無氮源）條件下，相較於野生型，TK 的生長不受影響。植物葉綠素主要為 Chl *a* 與 Chl *b* 兩種構型，Chl *b* 被認為主要來自 Chl *a* 構型轉化，若此假設成立，中間需經過去植醇反應。比較白化苗照光 4 小時內的葉綠素累積，Chl *b* 含量在 TK 中一開始略少但最終接近於野生型，此結果無法支持先前之假設。在黑暗誘導葉片老化過程，TK 與野生型之葉綠素降解程度並無顯著差別，表明 CLDs 並不參與此途徑。此外，TK 的生育酚含量顯著低於野生型，進一步分析不同組合的雙突變體，發現主要是因 CLD2 缺失造成，表明此酵素參與了生育酚的生合成。

關鍵字：

葉綠素去植醇酶、葉綠素轉換、葉綠素循環、葉綠素降解、生育酚合成

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Abstract

Chlorophyll is the basic element of photosynthesis. It participates in the biochemical reaction that converts light energy into biological energy to nourish most life on earth. Chlorophyll (Chl) contains hydrophilic chlorophyllide (chlorophyllide) and hydrophobic phytol (phytol) in structure, and the two parts can be hydrolyzed by dephytylation. Chlorophyll removal of phytol has been considered as a basic metabolic step of plants, participating in different chlorophyll-related metabolic pathways, including chlorophyll turnover, degradation, interconversion cycle and tocopherol biosynthesis. However, the enzymes are responsible for dephytylation mostly remained known. Recently, a novel chlorophyll dephytolase (CLD1) was discovered possibly involved in the chlorophyll turnover. The protein sequence analysis showed that CLD1 has homology with pheophytinase (PPH) involved in chlorophyll degradation during leaf senescence and two other proteins of unknown functions. Due to the latter two recombinant proteins have chlorophyll dephytase activity, they are further named as CLD2 and CLD3. In order to understand the physiological role of these phytolase enzymes, we established Arabidopsis cld1 cld2 cld3 (TK) and cld1 cld2 cld3 pph (QK) gene knockout mutants. Under normal and stress conditions (high light, or changes in luminosity, or high temperature, or nitrogen deficiency), the growth of TK is similar to that of wild-type. Plant chlorophyll mainly has two moieties of Chl a and Chl b. Chl b

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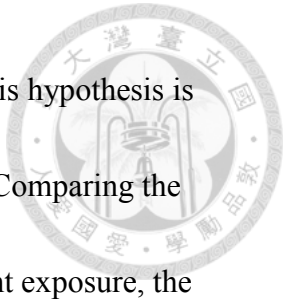

is considered to be mainly derived from the conversion of Chl a. If this hypothesis is established, the process needs to undergo a phytol removal reaction. Comparing the chlorophyll accumulation of etiolated seedlings within 4 hours of light exposure, the Chl b content in TK was slightly less at first but close to the wild-type in the end and cannot support the previous hypothesis. During the dark-induced leaf senescence process, there is no significant difference in the degree of chlorophyll degradation between TK and wild-type, indicating that CLDs do not participate in this pathway. In addition, the tocopherol content of TK was significantly lower than that of the wild-type. Further analysis of the double mutants of different combinations revealed that it was mainly caused by the lack of CLD2, indicating that this enzyme is involved in the biosynthesis of tocopherols.

Key words:

Chl dephytylase, Chl turnover, Chl cycle, Chl breakdown, tocopherol biosynthesis

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List of abbreviations:

ALA	5-aminolevulinic acid
CAO	chlorophyll *a* oxygenase
CBR	chlorophyll *b* reductase
Chl	chlorophyll
CHLG	chlorophyll synthase
Chlide	chlorophyllide
CLD1	chlorophyll dephytylase 1
CLD2	chlorophyll dephytylase 2
CLD3	chlorophyll dephytylase 3
CLH	chlorophyllase
CRISPR-Cas9	CRISPR-associated protein-9 nuclease
DILS	dark induce leaf senescence
GGPP	geranylgeranyl pyro(di)phosphate
GGR	geranylgeranyl reductase
HCAR	7-hydroxymethyl chlorophyll *a* reductase
HL	high light
HPLC	high performance liquid chromatography

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LHC	light harvest complex
LL	low light
MEP	methylerythritol 4-phosphate
NL	normal light
NYE	NON-YELLOWING
PAO	pheophorbide *a* oxygenase
Pchlide	protochlorophyllide
PDP	phytyl diphosphate
Pheide	pheophorbide
Phein	pheophytein
PPH	phephytinase
PS	photosystem
QK	*cld1-2 cld2-1 cld1-3 pph-1*
SGR	STAY-GREEN
TK	*cld1-2 cld2-1 cld1-3*
UPLC	ultra-performance liquid chromatography
Wt	wild-type (Columbia)

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Chapter 1. Introduction

Chlorophyll (Chl) enables photosynthetic organisms to capture sunlight to drive photosynthesis, which produces bioenergy that directly or indirectly fuels most life on earth. Chl molecule is composed of two moieties: a tetrapyrrole head and a phytol tail. The tetrapyrrole moiety offers electrons for photosynthesis under the excitation of sunlight, and phytol anchors Chl to the Chl-binding proteins in the two photosystems, photosystem I (PSI) and photosystem I (PSII). In plants and most cyanobacteria, Chl can be identified as Chl *a* and Chl *b*, the two predominant forms, and a less abundant form called pheophytin *a* (Phein *a*), which is structurally similar to Chl *a* but without the magnesium ion in the porphyrin. Chl *a* is present in all components of the photosystems, but Chl *b* is only present in the peripheral antenna, a complex composed of light-harvesting Chl-binding proteins (LHC). Phein *a* merely binds to D1 and D2 proteins of PSII core.

1.1. Chlorophyll *de novo* biosynthesis

Chlorophyll *de novo* biosynthesis starts from the condensation and polymerization of 5-aminolevulinic acid (ALA) into a cyclic tetrapyrrole and pauses after the generation of protochlorophyllide *a* (Pchlide *a*) when sunlight is not available

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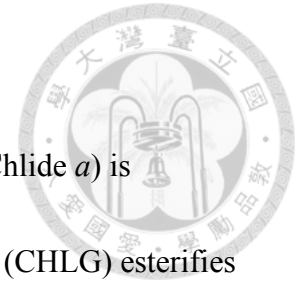

(Tanaka et al., 2011). Conversion of Pchlide *a* to chlorophyllide *a* (Chlide *a*) is light-dependent in angiosperm. Eventually, the chlorophyll synthase (CHLG) esterifies the Chlide *a* with either geranylgeranyl diphosphate (GGPP) or phytyl diphosphate (PDP) into the end product Chl *a* (Oster and Rüdiger, 1997), where the geranylgeranyl chain is reduced into phytol by geranylgeranyl reductase (GGR) before or after the esterification (Tanaka et al., 1999). For Chl *b* biosynthesis, Chl(ide) *a* oxygenase (CAO) drives the reaction by using Chl *a* or Chlide *a* as substrates. Previous study proposed direct conversion of Chl *a* to Chl *b* by CAO *in vivo* (Tanaka and Tsuji, 1981; Tanaka et al., 1995; Jia et al., 2016), but the recombinant CAO was shown to only oxidize Chlide *a*, not Chl *a*, *in vitro* (Oster et al., 2000; Rüdiger, 2002; Tanaka and Tanaka, 2011).

1.2. Chlorophyll cycle

It has been shown that Chl *b* can be synthesized in darkness, when the light-dependent conversion of Pchlide *a* to Chlide *a* is prohibited (Tanaka and Tsuji, 1981). Thus, it was proposed that Chl *b* can be converted from Chl *a* via a dephytylation step catalyzed by a Chl dephytylase (Jia et al., 2016). On the other hand, the conversion of Chl *b* into Chl *a* also plays an essential role in plant. Inhibition of this step causes stay-green phenotype in senescent leaf (Sato et al., 2009), affects seed maturation and

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storability (Nakajima et al., 2012), disturbs Chl turnover rate, and damages photosystems (Sato et al., 2015). Over accumulation of Chl *b* retards senescence and delays flowering time Sakuraba et al. (2012). Interconversion between Chl *a* and Chl *b* forms the Chl cycle (Tanaka and Tanaka, 2011). Chl cycle equips plants the ability to cope with varied light intensity in the nature, where excess light damages the photosystems and insufficient light diminishes photosynthesis efficiency. In response to low light, the antenna size of the photosystem is enlarged by generating more Chl *b* via Chl cycle. In contrast, under high light stress, the antenna size is decreased by converting Chl *b* to Chl *a* (Bjorkman et al., 1972; Leong and Anderson, 1984; Biswal et al., 2012). As Chlide *a* and Chlide *b* are involved in this cycle, again, the involvement of any Chl dephytylation in this pathway is still to be clarified.

1.3. Chlorophyll salvage

During steady state, as Chl turnover rate is much higher than its net loss, Chl turnover through a salvage route has been proposed (Matile et al., 1999; Ougham et al., 2008). In green tissue, Chl turnover is mainly associated with the damage of the photosystems, and the reaction rate is accordant to the intensity of environmental stresses. Among all the photosystem subunits, D1 protein is the most labile due to its

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role as a major electron acceptor in PSII reaction center, which is susceptible to photodamage. When D1 is damaged, it is degraded and replaced with a new functional one through PSII repair cycle (Autar K. Mattoo: Jonathan B. Marder, 1989; Mattoo et al., 1989; Sundby et al., 1993; Nath et al., 2013). In this process, the Chls bound to the damaged D1 should be temporally deposited to avoid phototoxicity and possibly reused (Jutta Papenbrock, 2000; Papenbrock et al., 2000; Meskauskiene et al., 2001; Pruzinska et al., 2003; Hirashima et al., 2009). Indeed, an isotope tracing experiment executed in cyanobacteria showed that Chls detached from the D1 protein were reused through dephytylation and rephytylation steps (Vavilin and Vermaas, 2007). In Arabidopsis, through the study of a heat sensitive mutant, our lab demonstrated that CHLG catalyzes the rephytylation step (Lopez et al., 1996; Lin et al., 2014). The mutant, named *chlg-1*, has a point mutation in the coding region of CHLG and maintains only 10% of CHLG protein at normal condition as compared with that in Wt (wild-type, Columbia). After heat stress (40°C for 1 h), CHLG protein decreases by about 50% in both Wt and *chlg-1*, and substantial increase of Chlide *a* can be detected in *chlg-1* but not Wt. Accumulation of Chlide *a* causes severe photodamage of *chlg-1* under light condition. Since D1 is the only protein decreased in the survey of most photosystem subunits, it was proposed that the heat-induced surge of Chlide *a* in *chlg-1* should be derived from the dephytylation

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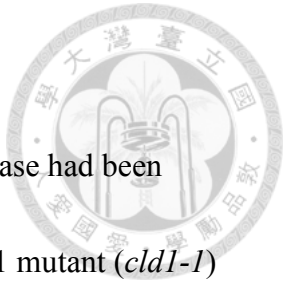

of Chl *a* released from the damaged D1 proteins. A new Chl dephytylase had been identified in 2016 as CLD1 through its supraoptimal activity of CLD1 mutant (*cld1-1*) (Lin et al., 2016). *cld1-1* showed a significantly increase in Chlide *a* after in vitro dephytylase activity test and heat treatment performed in seedlings. The *cld1-1 chlg-1* double mutant accumulated more Chlide *a* than in the *cld1-1*, indicating that there may be a salvage cycle between CLD1 and CHLG. However, the phylogenetic analysis of CLD1 demonstrated that other three homologs existed in Arabidopsis, suggesting that there are additional Chl dephytylases may play a role in Chl salvage cycle (Lin et al., 2016).

1.4. Chlorophyll breakdown

Massive Chls loss through Chl dephytylation during leaf senescence had been proposed for a century, but proven wrong until recently. In the past, a hydrolase named chlorophyllase (CLH) was considered to be involved in Chl degradation during leaf senescence based on its Chl dephytylation activity *in vitro* (Debora Jacob-Wilk and Eliezer E. Goldschmidt1, 1999; Tsuchiya et al., 1999; Takamiya et al., 2000; Hortensteiner, 2006; Morita et al., 2009). However, the reverse genetic studies in Arabidopsis showed that *CLH* genes are not required for leaf senescence (Schenk et al.,

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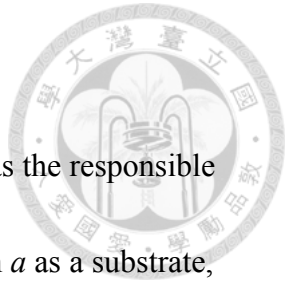

2007; Hu et al., 2015). Instead, pheophytinase (PPH) was identified as the responsible enzyme. PPH has *in vitro* dephytylation activity specifically on Phein *a* as a substrate, and its knockout lines showed stay-green phenotype and Phein *a* retention during leaf senescence (Schelbert et al., 2009). To date, the pathway leads to Chl breakdown is largely clear. It starts with the conversion of Chl *b* to Chl *a* by Chl *b* reductase (CBR) and 7-hydroxymethyl Chl *a* reductase (HCAR), and then Chl *a* is dechelated to form Phein *a* by the Mg-dechelatase (NON-YELLOWING1, NYE1 or STAY-GREEN, SGR) (Armstead et al., 2007; Sato et al., 2007; Shimoda et al., 2016; Li et al., 2017). Phein *a* is dephytylated by PPH to generate Pheide *a*, followed by cleavage of the porphyrin ring by Pheide *a* oxygenase (PAO). The product of PAO is then further processed by the PAO/phyllobilin pathway (Collakova and DellaPenna, 2003; Ischebeck et al., 2006; Vom Dorp et al., 2015), (Hortensteiner, 2009, 2013; Shimoda et al., 2016). However, the *PPH*-deletion mutant still shows substantial loss in Chl content during the dark-induced leaves senescence (Schelbert et al., 2009). Furthermore, PPH was shown to be dispensable for Chl breakdown during seed maturation and tomato fruit ripening (Guyer et al., 2014; Zhang et al., 2014). These results suggest that there may be other dephytylase(s) participating in Chl breakdown pathway.

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1.5. Chlorophyll dephytylation and tocopherol biosynthesis

Tocopherols are important antioxidants for plant tolerance to oxidative stress, and they are also important for human diet in a name better known as Vitamin E. Tocopherols are potent lipid soluble antioxidants, containing a chromanol ring with a saturated phytyl side chain. Their function is exemplified in planta by the demonstrated role in promoting seed longevity by reducing the accumulation of lipid oxidation products during storage (Sattler et al., 2004). Tocopherols are only synthesized in photosynthetic organisms (DellaPenna and Mène-Saffrané, 2011).

In general, the tocopherol content in the photosynthetic tissues is low comparing to the high level in seeds, (10-50 vs 300-2000 μg/g FW) (McLaughlin and Weihrauch, 1979; Grusak, 1999). In plant, four tocopherol isoforms can be found: α, β, γ, and δ. In *Arabidopsis* leaves, α-tocopherol is the major isoform, but in seeds, γ-tocopherol is predominant (DellaPenna and Mène-Saffrané, 2011). A tocopherol molecule is formed by conjugating a homogentisate head from cytosolic shikimate pathway and a hydrophobic tail from phytyl diphosphate (PDP) from plastid methylerythritol phosphate pathway (MEP pathway) (Lushchak and Semchuk, 2012). PDP can originate from direct reduction of geranylgeranyl diphosphate (GGPP) by geranylgeranyl diphosphate reductase (GGR) (Kuntz et al., 1992; Hugh A. Addlesee, 1996; Yves

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KELLER, 1998; Addlesee and Hunter, 1999; Okada et al., 2000). Alternatively, it can also be generated by consecutive phosphorylation of phytol presumably released from Chl or Pheine *a* via dephytylation (Ischebeck et al., 2006). Phytol, which occupies approximately one-third of the mass of Chl, is considered to be the most abundant acyclic isoprenoid compound in the biosphere (Volkman, 1986).

It has been shown that chlorophyll-derived phytol is the major precursor for tocopherol biosynthesis based on the genetic studies of Arabidopsis mutants of phytol kinase and phytol kinase kinase (Vom Dorp et al., 2015). The phytol kinase is named VTE5 (At5g04490), and tocopherol content is decreased in v*te5* mutant up to 50% (Valentin et al., 2006; Vom Dorp et al., 2015). The phytol phosphate kinase is named VTE6 (At1g78620), and the α-tocopherol amount of *vte6* is more significantly decreased (~95%). Hence, phytol released by Chl dephytylation is considered the main source for tocopherol biosynthesis. However, the responsible dephytylase is still unknown as the suppression or over-expression of either PPH or CLD1 did not obviously change the tocopherol content (Zhang et al., 2014; Lin and Charng, 2017).

1.6. Research goal

According to the phylogenetic analysis, there exist another two homologs of *CLD1*

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and *PPH* in *Arabidopsis thaliana* genome (Lin et al., 2016), named *CLD2* (At4g36530) and *CLD3* (At5g19850) in this study. Previously, *in vitro* enzyme activity assay showed that both the recombinant CLD2 and CLD3 can hydrolyze Chl*a*, Chl *b*, and Phein *a* in different ability (Supplemental Figure 1, Lin et al., unpublished data), suggesting that CLD2 and CLD3 may have redundant function to CLD1 and PPH. In this study, we generated the double knockout (DK) and triple knockout (TK) mutants with different combination of *CLD1*, *CLD2*, and/or *CLD3* deletion through genetic crossing. The quadruple knockout (QK) mutant with the deletion of *PPH* and all three *CLD* genes was generated as well. The homeostasis of Chl metabolism and tocopherol content in these mutants under normal and different stress conditions were investigated to characterize the physiological function of CLDs.

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Chapter 2 Material and Methods

2.1. Plant materials

The KO mutants of Arabidopsis *CLD*s used in this study are all in Col-0 background. *CLD1* KO lines, *cld1-2* and *cld1-3* were generated previously by using CRISPR-Cas9 system (Lin YP, unpublished data)(Wang et al., 2015). *cld2*-1 (SALK-_148524) and *cld3-1* (SAIL_402_G10) were obtained from Arabidopsis Biological Resource Center. *pph-1* (SALK_000095) was kindly provided by Dr. Stefan Hörtensteiner (University of Zürich, Switzerland). Double (*cld1-2 cld2-1*, cld*2-1 cld3-1*, cld*1-2 cld3-1*), tiriple (*cld1-2 cld2-1 cld3-1*), and quadruple (*cld1-2 cld2-1 cld3-1 pph-1*) KO mutants were generated by crossing of the single mutants. Seeds were sown on plates containing 0.8% agar with half-strength Murashige-Skoog medium and 0.1 or 1 % sucrose, imbibed for 3 d at 4°C in the dark for seed stratification. Then, the plates were incubated at 22°C with a 16-h/8-h light-dark cycle (80 μmol $m^{-2}s^{-1}$ light intensity), which is considered the standard condition in this study.

2.2. Genotyping

Seedlings (about 20~30 mg) frozen in liquid N_2 were homogenized with a homogenizer (SH-48) according to the manufacturer's instruction .

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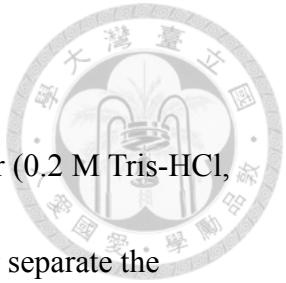

Ground tissues were mixed with the 400 μL of DNA extraction buffer (0.2 M Tris-HCl, pH7.5, 0.25M NaCl, 25 mM EDTA, and 0.5% (v/v) SDS) and further separate the protein by 500 μL phenol/chloroform/isopropanol (25:24:1). Equal amount of the isopropanol was added to the supernatant after the centrifugation of 16,200 x g at room temperature. DNA was pelleted by 16,200 x g at 4°C and wash by 70% (v/v) EtOH for twice, then dried pellet was dissolved in 20 μL of ddH_2O water. The concentration of DNA was determined by NanoDrop 1000 Spectrophotometer.

DNA was amplified by PCR with the setting in Table 1. Primer set of each mutant were shown in Table 2. PCR product was confirmed in 1 % agarose gel and detected by GelDoc XR+ (Bio-Rad).

2.3. Stress treatments

For heat stress, five-d-old seedlings grown on a plate containing 0.8% agar with half-strength Murashige-Skoog medium and 0.1 % sucrose were treated at 35°C during the day and 33°C during the night for 7 d with a 16-h/8-h light-dark cycle and recovered at the standard condition for another 7 d. For light stress, high light (HL, 650 μmol m^{-2} s^{-1}) and fluctuating light (FL) treatments were applied using an LED white light panel (High Point) immediately after the end of seed stratification. During light stress, the

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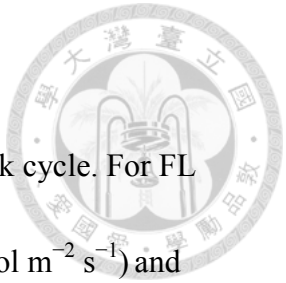

plants were maintained at room temperature with a 16-h/8-h light-dark cycle. For FL stress, light intensity was set at alternate 1-min of high light (650 μmol m^{-2} s^{-1}) and 5-min of low light (10 μmol m^{-2} s^{-1}) during the light cycle for 11 d. For nitrogen deficiency, 3-d-old seedlings grown on regular agar medium with 1% sucrose were transferred to the medium without sucrose and nitrogen for 7 d.

2.4. Dark-induced leaf senescence

Treatment was similar to previous described (Schelbert et al., 2009). Rosette leaves were detached from 5~6 weeks plants and placed on a filter paper moistened with 3 mM MES buffer in a plate. The plate was sealed with 3M micropore, wrapped with aluminum foil to prevent light, and incubated at 22°C for 5 d. Leaf senescence was judged by the degree of yellowing and the Chl level of the detached leaves.

2.5. Seeds accelerated aging test

Seeds stored for three months at 17°C (RH 21 %) after harvest were used for accelerated aging test basing on the wire-mesh method (Sattler et al., 2004) with modifications. Seeds were placed above 400 mL of water on the plastic round disc in boxes (14 cm x 14 cm x 4.5 cm) with wet filter paper and plastic wrap sealed. 100 seeds

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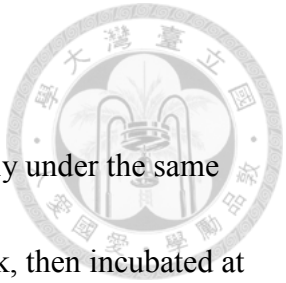

were used for each genotype and all plants were grown simultaneously under the same conditions. Seed were aged for 72 h at 40°C and 100% RH in the dark, then incubated at 4°C for 5 d to break dormancy. Seeds without aging (controls) were also incubated on the moistened filter paper at 4°C for 5 d, then seeds were placed at 22°C under a 16-h/8-h light-dark cycle (100 μmole$m^{-2}s^{-1}$ light intensity). Seed germination rate was determined by counting the number of seeds with root radical emergence every day up to 10 d.

2.6. Determination of chlorophyll, tocopherol

Chlorophyll, tocopherol, and free phytol were measured at different developmental stages, including mature seeds, 9-d or 11-d-old seedlings, and rosette leaves. For extraction of chlorophyll and tocopherol, 50 mg of fresh tissues (seedlings and rosette leaves) or 5 mg of seeds frozen in liquid nitrogen were ground to a fine powder with grinding beads (Kurabo). The fresh tissue and seed powders were re-suspended in 500 μL and 200 μL, respectively, of alkaline acetone solution (90% acetone, 0.2 M NH_4OH) that had been pre-cooled at -20°C. After vortex, the mixture was incubated on ice for 10 min and centrifuged at 16,200xg at 4°C for 10 min. The supernatant was transferred to a new Eppendorf tube, and 200 μL of it was subjected for ultra performance liquid

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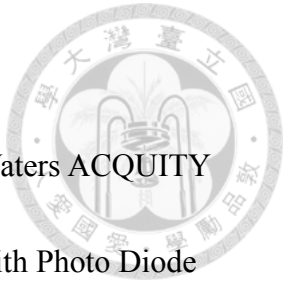

chromatography (UPLC) analysis. Chlorophylls were separated by Waters ACQUITY UPLC BEH C18 column (1.7 μm particle size, 2.1 mm x 100 mm) with Photo Diode Array Detector(PDA) absorption wavelength of 410 nm (Chl *a*) and 450 nm (Chl *b*), while tocopherols were separated by Waters ACQUITY UPLC BEH C8 column (1.7 μm particle size, 2.1 mm x 100 mm) and monitored excitation/emission at 290 nm/330 nm with Fluorescence (FLR) Detector.

2.7. Subcellular localization of CLDs

Fractionation of chloroplast compartments was performed as previously described (Chu and Li, 2011). Briefly, leaves of 4 to 5-week-old plants were ground with the isolation buffer (20 mM Tricine-KOH, 0.45 M sorbitol, 10 mM EDTA, 10 mM $NaHCO_3$, 0.1% BSA pH 8.4). The ground sample was filtered through Miracloth. The filtrate was centrifuged at 500 x g (Acc max/ Decc slow) for 20 min at 4°C to collect pellet, which was then resuspended in the resuspension buffer (100 mM Tricine-KOH, 1.5 M sorbitol, 10 mM EDTA, 25 mM $MgCl_2$, pH 8.4). The suspension was loaded onto the Percoll step gradient (40% [v/v] and 80% [v/v]) and followed by centrifugation of 6500 x g (Accel slow/ Decc off) at 4°C. The interface between Percoll gradient was collected, resuspended and washed with the resuspension buffer twice. Finally, the suspension was

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centrifuged at 3800 x g for 6 min at 4°C. The pellet was resuspended with moderate amount of the resuspension buffer (1 g FW:100 μL buffer). Concentration of chloroplast (mg chloroplast ml^{-1}) was determined through OD_{652} by BioTek Synergy H1 with 2Di.

Chloroplasts were further fractionated into envelope/stroma and thylakoid fractions by freeze and thaw with liquid N_2 for third rounds. The solution was centrifuged at 3000 x g for 3 min at 4°C. The envelope/stroma and thylakoid fractions are located in the supernatant and pellet, respectively.

2.8. Protein extraction and immunoblotting

Total proteins were extracted from 4 to 5-week-old Arabidopsis plants and used for immunoblotting as described previously (Charng et al., 2006). Protein content was quantified using the BioTek Synergy H1 with 2Di with BSA as standard. For immunoblot analysis, an SDS-PAGE minigel (NuPAGE 4-12% BisTris gel system; Invitrogen) was used for separation, then transferred to a nitrocellulose membrane for immunodetection using chemiluminescence reagent (Western Lighting Plus-ECL; PerkinElmer). The polyclonal antiserum against Arabidopsis CLD2 and CLD3 were produced by immunizing rabbits. Synthetic peptides (CLD2, N'-WRYNIPELAKKYKVYALC; CLD3, N'-WGEKDPWEPIELGRAYSNC) were

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performed by LTK Biotechnology (http:// www.ltk.com.tw/). The antibodies against CLD1, Rbcl and Lhcb1 were described previously (Lin et al., 2016). After incubated in secondary antibody for 1 h, luminescent signals were detected by BioSpectrum 815 imaging system (UVP). The membrane was stained with Ponceau S (0.1%, w/v) for ensuring equal protein loading.

2.9. Accession numbers of genes

The Arabidopsis Genome Initiative (AGI) numbers of the genes mentioned in this work are: At5g38520 (*CLD1*), At4g36530 (*CLD2*), At5g19850 (*CLD3*), and AT5G13800 (*PPH*).

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Chapter 3 Results

3.1. Generation and isolation of *CLD*s and *PPH* knockout mutants

To investigate the physiological functions of the CLD isoforms, different knockout lines with single or multiple *CLD* homologs were generated. The *CLD1*-knockout (KO) mutants were generated by CRISPR-Cas9 method (Lin YP, unpublished data), and two independent lines, *cld1-2* and *cld1-3*, were isolated. The mutant *cld1-2* has an extra cytosine at position 399 (the adenine of the start codon is assigned as position 1), which causes a premature stop codon at 409 while the other mutant *cld1-3* has a deletion between exon1 and exon2 (89-531). Here, we only focused on *cld1-2*. *cld2-1* and *cld3-1* are mutants with a T-DNA insertion in the intron 1 and exon 7, respectively. The mutant *pph-1* is also a T-DNA insertion mutant reported previously (Schelbert et al., 2009) (Figure 1a). *pph-1* does not show apparent difference under the normal condition when comparing to the Col-0 (wild type) before senescence (Poudyal et al., 2020). However, during senescence, *pph-1* shows a stay-green phenotype (Schelbert et al., 2009; Zhang et al., 2014). The double mutants (DK) with different combination of *cld1-2*, *cld2-1*, and *cld3-1*, were generated by crossing each other and confirmed by genotyping (Figure 1b). *cld1-2 cld2-1 cld3*-1 triple mutant (TK) and *cld1-2 cld2-1 cld3-1 pph-1* quadruple mutant (QK) were also generated subsequently. Under normal condition with 16 h of

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light (80 nmol m^{-2} s^{-1}) per day, TK showed growth and development comparable to that of the Wt (Figure 2e), suggesting that CLDs are dispensable for plant growth and development.

3.2. CLD2 and CLD3 are associated with thylakoid

The CLD1 localization had been confirmed to be associated with the thylakoid in the previous study (Lin et al., 2016). To further investigate the role of other two CLDs, their localization was examined. The results of fractionation of the intact chloroplasts show that both CLD2 and CLD3 were present in the thylakoid fraction (Figure 1c), but not in the stroma, which is the same as CLD1. The antibodies against CLD2 and CLD3 are quite specific as the band signals were not detected in the corresponding KO mutants. The results confirm that all three CLDs are located within the chloroplasts.

3.3. Functional studies of CLD isoforms under stress conditions

Suppression of CLD1 activity by RNAi was shown to compromise the thermotolerance to moderately high temperature (TMHT) in Arabidopsis seedlings (Wu et al., 2013; Lin et al., 2016), suggesting that Chl dephytylase activity is required for stress tolerance. However, *cld1-2* KO mutant did not show heat sensitive phenotype like

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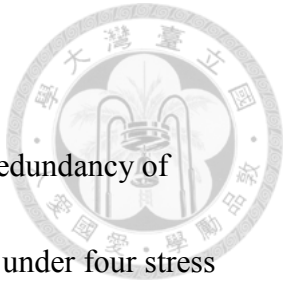

the RNAi lines (Lin et al., unpublished results). To avoid functional redundancy of CLDs, *cld1-2 cld2-1 cld3-1* was employed for the physiological tests under four stress conditions, *i.e.*, high light, heat stress, nitrogen deficiency, and fluctuating light.

In the field, high light is the most common abiotic stress factor that plant encounters frequently. High light intensifies the damage and degradation of D1 protein, and the bound Chls should be recycled after releasing from D1 (Raskin et al., 1995). Because Chl dephytylation and rephytylation were shown to involve in Chl recycle (Vavilin and Vermaas, 2007), it is likely that at least one of the CLDs is involved in this process. Under high light condition, if Chls were not recycled properly or there is a defect in photoprotection gene, the excited free Chls can lead to singlet oxygen accumulation, and the photodamage will decrease the photosynthetic activity, then eventually retard growth. To see if the mutant devoid of CLDs' functionalities was more sensitive to high light stress than wild type, high intensity of light (650 μmol m^{-2} s^{-1}) was applied to the young seedlings for 11 d. The growth of the seedlings under the high light condition was severely affected as compared with those grown under standard condition. High light stress resulted in smaller plant size with pale-green leaves (Figure 2a). However, there was no obvious difference between TK mutant and wild type (Figure 2a), suggesting that CLDs are not required for tolerance against high light stress

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under the tested condition.

During HS treatment in laboratories, which was done in the dark, plant stops the Chl *de novo* biosynthesis and heat-stringency accordant loss of D1 protein content followed by the Chlide accumulation occurred (Lin et al., 2016). To investigate if any CLD homolog is involved in this pathway, the growth of Wt and TK under thermotolerance to moderately high temperature (TMHT) was compared. The 5-d-old seedlings growing under normal condition were moved to the chamber with 35°C (16 h, day)/33 °C (8 h, night) for further 7 d. After recovery for another 7 d at normal condition, no clear thermotolerance defect was identified in TK (Figure 2b).

Nitrogen is a vital element for porphyrin biosynthesis. Nitrogen deficiency will lead to the accumulation of starch, lower level of CO_2 and Chl content in plant (Cave et al., 1981; Boussadia et al., 2010). Without the nitrogen supply, to maintain the Chl content, plants may undergo the de- and rephytylation for saving the nitrogen. To see if the CLDs were involved in recycling of the nitrogen by dephytylation, Arabidopsis seeds were sowed on the agarose plate with 1/2 MS and 1 % sucrose and grown for three day under normal condition before transferring to the nitrogen and sucrose free medium. After another 7-d growth under nitrogen deficient condition, both Wt and TK showed similar growth (Figure 2c).

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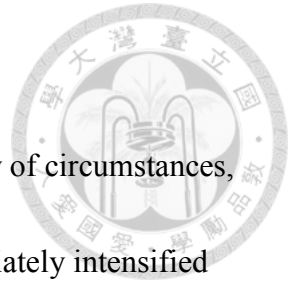

In nature environment, fluctuating light (FL) occurred in variety of circumstances, for instance, shadowing by neighboring plants and cloud. The immediately intensified light can lead to the reduction of PSII activity and a state transition between photosystems. Under FL condition, due to the D1's labile characteristic, PSII supercomplex has to be repaired by degrading D1 and recycling Chls. Previous studies showed that mutants of the genes involved in the photoprotection, photosystem state transitions and light adaptation exhibited stunted growth phenotype under FL conditions (Grieco et al., 2012; Suorsa et al., 2012; Bhuiyan et al., 2015; Hou et al., 2015). To see whether CLDs also involved in this process, the Wt and TK plants were subjected to FL treatment for 11 d after seed germination. The FL treatment caused severe stress to the plants, which were stunted in comparison to the control (Figure 2d). No obvious phenotypic difference was observed between Wt and TK plants.

Based on the above four assays under different stress conditions, the results suggest that CLDs are dispensable for plant growth under the tested conditions.

3.4. Functional studies of CLD isoforms in Chl *b* biosynthesis during photomorphogenesis

It has been hypothesized that Chl *b* is synthesized by converting the pre-existing

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Chl *a* via dephytylation, oxidation, and esterification (Tanaka and Tsuji, 1981; Jia et al., 2016). If this hypothesis is correct, a Chl dephytylation step driven by at least one of the three CLDs is required, and disruption of the responsible CLD should block Chl *b* biosynthesis. To test this hypothesis, Chl *b* content was analyzed in Wt and TK seedlings grown under different light intensity, which may alter the Chl *b* content and consequent Chl *a*/*b* ratio (Biswal et al., 2012). Seedlings were grown under HL (650 μmol m^{-2} s^{-1}), NL (80 μmol m^{-2} s^{-1}), LL (30 μmol m^{-2} s^{-1}) for 11 d with 16 h-light per day. The result showed that increase in light intensity decreased the Chl *b* content and raised Chl *a*/*b* ratio in either Wt or TK. Under the same condition, TK showed no significant difference in Chl *b* level as compared to Wt (Figure 3a and 3b).

Secondly, the Chl *b* content in greening seedlings was also investigated. After 4 d of dark incubation, the etiolated seedlings were transferred to the LL condition, and Chls were analyzed at different time point. Chl *a* contents in TK and Wt were comparable. However, TK had a Chl *b* content 30% lower than Wt at the first hour. No significant difference in Chl *b* content between TK ad Wt plants after prolonged illumination (Figure 3c, 3d). Chl *a*/*b* ratio also showed the similar result with a higher ratio at the first hour in TK and gradually decreased to the Wt level afterward (Figure 3e). This result suggests that Chl *a* dephytylation catalyzed by CLDs only play a minor

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role in the biosynthesis of Chl *b* in greening seedling at a very early stage.

3.5. Functional studies of CLD isoforms in Chl breakdown pathway

PPH was demonstrated to be a dephytylase of Phein *a*, not Chls, and is required for Chl breakdown during leaf senescence (Schelbert et al., 2009). However, recent studies suggested the existence of alternative dephytylase(s) involved in Chl breakdown as well (Lin and Charng, 2021). We wondered whether CLDs are involved in this process given their Phein *a* dephytylation activity *in vitro* (Supplemental Figure 1). To address this question, we performed a dark-induced leaf senescence (DILS) assay to compare the Chl degradation rate in Wt, TK, QK, and *pph-1* mutants. Five to 6-wk-old plants were used in this experiment. The 7^{th} to 10^{th} leaves, counted from the bottom to the top of the rosette, were detached and subjected to 5-d incubation in the dark. The results showed that *pph-1* had a stay-green phenotype, which is consistent with previous studies. However, there was no difference between Wt and TK (Figure 4a, 4b). Analysis of Chl in the senescent leaves showed that Wt and TK equally lost massive amount of Chl *a*, -Chl *b*, and Phein *a*, and QK maintained as much of the pigments as in *pph-1* (Figure 4c-4e). These results suggest that CLDs are not required for Chl breakdown during leaf senescence.

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3.6. Functional studies of CLD isoforms in tocopherol biosynthesis

It was proposed that the free phytols released from Chls are recycled to form Chls and tocopherols (Vom Dorp et al., 2015; Zhang et al., 2015). Hence, if CLDs were involved in tocopherol biosynthesis by generating phytol via Chl dephytylation, one would expect reduction of tocopherol level in the mutants devoid of responsible CLD activity. To see if the CLDs are participated in the toopherol biosynthesis pathway. First, the tocopherol levels in the green and senescent leaves were measured for Wt, TK, QK, and *pph-1* before and after the DILS treatment in adult plant. The results show that no significant difference in α-tocopherol was observed neither between Wt and TK nor between QK and *pph-1* before and after DILS (Figure 5a), suggesting that the three CLDs are not involved in α-tocopherol biosynthesis in these tissues. However, *pph-1* had a much lower level of α-tocopherol than in Wt in both the green and senescent leaves (Figure 5a), suggesting that pheophytin *a* dephytylation catalyzed by PPH is required for the biosynthesis of a significant portion of α-tocopherol.

Furthermore, the tocopherol content in seed and seedling was also analyzed to clarify the involvement of CLDs. The γ-tocopherol content in the mature seeds was the same between Wt and TK, suggesting CLDs are not required for γ-tocopherol

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biosynthesis in the seeds. About 10 % reduction of γ-tocopherol was found in QK seeds as compared to that of Wt and TK (Figure 5b), suggesting that this reduction is mainly contributed by the *pph-1* allele. Meanwhile, in the whole seedlings, α-tocopherol was significantly decreased by 20% in both TK and QK as compared to Wt (Figure 5c), suggesting that CLDs play a minor role in tocopherol biosynthesis at seedling stage. To identify which CLD isoform participates in α-tocopherol biosynthesis in the seedlings, theα-tocopherol contents in the double mutants, *cld1-2 cld2-1*, *cld2-1 cld3-1*, and *cld1-2 cld3-1* were compared. The results show that the double mutants carrying *cld2-1* allele had tocopherol level as low as that in TK, but not in the *cld1-2 cld3-1* (Figure 5d), suggesting that, among the three CLDs, CLD2 plays a more important role in tocopherol biosynthesis in the seedlings.

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Chapter 4 Discussion

Chls are important pigments that play a pivotal role in photosynthesis. Either deficient or over accumulation of Chls in plants could lead to disadvantage in growth. The dephytylation of Chl is considered to be involved in Chl catabolism, but its physiological role and the responsible enzymes remain unclear.. The newly identified Chl dephytylase, CLD1, and its homologs, CLD2 and CLD3, serve as good candidates in addressing this issue. First, CLD2 and CLD3 were shown to localize in the thylakoid (Fig. 2c), which is the same as CLD1 (Lin et al. 2016). By using a reverse genetic approach, I examined relevant physiological and biochemical responses of the Arabidopsis mutant devoid of all three CLD homologs.

4.1. The role of CLDs in response to abiotic stresses

Chl dephytylation is thought to involve in Chl turnover, and abiotic stresses that cause damage to photosystems should accelerate Chl turnover during the process of photosystem repair. Hence, blocking Chl dephytylation would exacerbate damage of photosystems. In this study, TK (knocking out all three CLD-isoforms) and QK (TK plus *pph-1*) were generated and showed comparable growth to Wt under normal and the tested stress conditions (Figure. 2). Under the stress conditions of high temperature,

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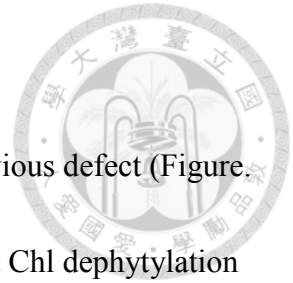

highlight, fluctuating light, or nitrogen-depletion, TK showed no obvious defect (Figure. 2). These results suggest two possibilities. The first possibility is that Chl dephytylation is not the sole pathway for Chl turnover. The ability of cyanobacteria to directly excrete extra tetrapyrrole to the medium implies another Chl catabolic pathway without Chl dephytylation (Fujita et al., 1992). The second possibility is that there may be other dephytylase(s) involved. Such possibility is supported by a recent studies in CLHs and cyanobacteria. Recently, there's a report suggesting that the Arabidopsis chlorophyllases, CLH1 and CLH2 were localized in the thylakoid at the new growing young leaf and it will not be visible in chloroplast but cytosol by the leaf maturation. Moreover, they also proposed that CLHs were the dominant Chl dephytylase in high light stress induced Chl turnover (Tian et al., 2021). In cyanobacteria, a novel protein Slr1916 was shown to have in vitro Chl dephytylase activity (Chen et al., 2021). By BLASTP searching of Arabidopsis protein database, Slr1916 showed highest score in homology to PHYLLO (AT1G68890), a phylloquinone biosynthetic enzyme (Gross et al., 2006). PHYLLO also contains a chloroplast transit peptide and conserved lipase motif (Ser active site), suggesting that it may serve as a novel Chl dephytylase (Tsuchiya et al., 1999). So, it cannot be excluded that these Chl dephytylases are functioning independently or redundantly with the CLDs. Although TK survived as well as the Wt under the stress

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conditions tested in this study, some other environmental stimuli that affect photosystem/Chl turnover rate are worth of further investigation, including cold (Gong et al., 2014), salt (Demetriou et al., 2007), or the combination challenge such as heat plus highlight (Spicher et al., 2017).

4.2. The role of CLDs in Chl cycle in Arabidopsis seedlings

It is still an open question whether a dephytylation step is required for the conversion of Chl *a* to Chl *b* in the Chl cycle poposed previously (Tanaka and Tsuji, 1982) . As demonstrated in previous studies, plants grown under low light produce more Chl *b* and have a lower Chl *a*/*b* ratio in comparison with those grown under high light (Bjorkman et al., 1972; Leong and Anderson, 1984; Biswal et al., 2012). In this study, no difference in Chl *b* content and Chl *a*/*b* ratio was observed between Wt and TK while plants were grown under both high and low light conditions (Figure 3a and 3b), suggesting that Chl dephytylation step may not participate Chl *b* biosynthesis in Chl cycle or that another unidentified Chl dephytylase is responsible.

Chl *a* plays a vital role in photosynthesis whereas Chl *b* is dispensable in higher plants (Tanaka and Tanaka, 2011). It was shown that upon exposure of the etiolated seedlings to the light Chl *a* is first generated, then converted Chl *b* (J. H.

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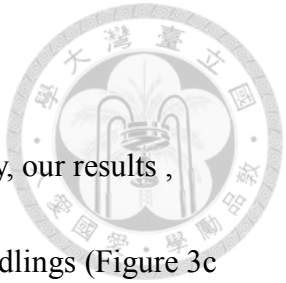

Argyroudi-Akoyunoglou, 1976; Tanaka and Tsuji, 1982). In this study, our results , reveal the function of CLDs in Chl *b* biosynthesis of the greening seedlings (Figure 3c and 3e). The disruption of CLDs reduced Chl *b* level at the first hour of light exposure, but not at further time points, suggesting that CLDs play a role in the initial conversion of Chl *a* to Chl *b*. However, CLDs are not essential for Chl *b* biosynthesis.

4.3. The role of CLDs in Chl breakdown

PPH is responsible for Chl breakdown in senescent leaves, but not in maturing seeds and fruits, suggesting the existence of alternative dephytylase (Lin and Charng, 2021)). In Chl breakdown, Phein *a* instead of Chls is the substrate of PPH. In addition to PPH, CLDs are capable of hydrolyzing Phein *a* (Supplemental Figure 1). Hence, it is likely that these CLDs are involved in Chl breakdown. This notion is supported by the CLDs gene expression profile in Arabidopsis as shown by the transcriptome data visualized with the eFP browser (Figure 6). Both *CLD2* and *CLD3* are expressed in the senescent leaves. However, our results suggest that CLDs are not required for Chl breakdown during dark-induced leaf senescence (Figure 4). Since Chl breakdown still occurred to some extent in the senescence leaves of *pph-1* (Figure. 5), Chl might be degraded via other pathway independent of these four known dephytylases. For

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example, senescence associated vacuoles (SAVs) or chloroplast vesiculation may also participate in the degradation of Chls (Martinez et al., 2008; Wada et al., 2009; Wang and Blumwald, 2014; Gomez et al., 2019).

4.4. The role of CLDs in tocopherol biosynthesis

Previous works suggest that phytol detached from Chl is the major source for tocopherol biosynthesis (Ischebeck et al., 2006). If this is the case, suppression of Chl dephytylation should dramatically reduce the amount of tocopherol as is the case in *vte5* and *vte6* mutants (Vom Dorp et al., 2015). However, our results showed that Arabidopsis mutants devoid of three CLDs could synthesize α-tocopherol up to 75% of the wild-type level in the young seedlings (Figure 5c). This result indicates that the CLDs play a role in α-tocopherol biosynthesis at least at young seedling stage. PPH seemed to be dispensable at this stage because no further reduction of α-tocopherol in QK as compared with the TK mutant (Figure 5c). On the other hand, the substantial reduction of α-tocopherol was observed in QK and *pph-1* mature leaves, but not in TK (Figure 5a), suggesting that PPH, but not CLDs, is involved in tocopherol biosynthesis in mature leaves. Interestingly, no significant change in γ-tocopherol was observed in TK mature seeds, while 10 % reduction was found in QK (Figure 5b), suggesting that

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PPH, but not CLDs, plays a role in γ-tocopherol biosynthesis in the mature seeds.

In this study, the results showed that a major portion of tocopherols in all tested tissues were not dependent on the activities of CLDs and PPH. Hence, biosynthesis of these tocopherols may require dephytylation of Chls catalyzed by unidentified dephytylases. Although CLHs were recently shown to be localized in chloroplast (Tian et al., 2021), their role in tocopherol biosynthesis has been excluded genetically previously in developing seeds and mature seeds (Zhang et al., 2014). Alternatively, the GGPP pathway may be reduced by GGR to form PDP by feedback regulation when free phytol supplied from Chl dephytylation is limited. However, this hypothesis is not supported by the works of Dormen's group on *vte5* and *vte6* mutants (Vom Dorp et al., 2015). More effort is required to provide better insight into this issue.

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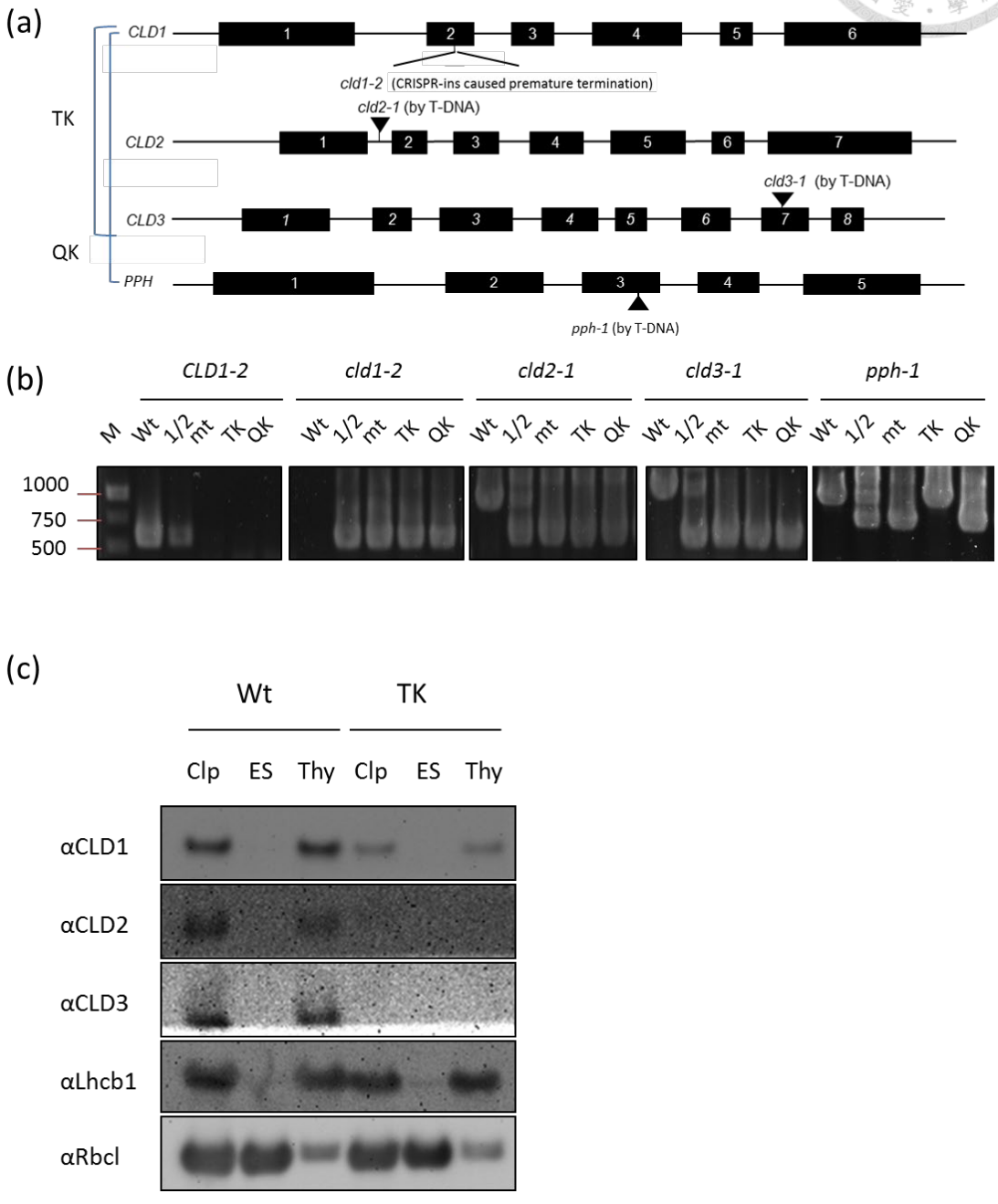
Figures



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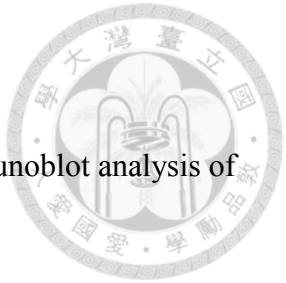

Figure 1. Gene structures of *CLDs*, *PPH* and mutant alleles and immunoblot analysis of CLDs protein localization.

(a) Gene structure and the site of CRISPR-Cas9 and T-DNA insertion.

(b) Genotyping analysis of *clds* triple and *clds pph-1* quadruple knockout mutant. PCR products are examined by 1% agarose gel and visualized with EtBr. The "1/2" label indicates the heterozygous mutant and "mt" indicates the homozygous single mutant. Upper panel of gel image demonstrate the primer we use. CLD1-2 and cld1-2 primers can recognize the Wt and mutant alleles, respectively.

(c) Localization of CLDs. Intact chloroplasts (Clp) isolated from 4-week-old plants and further separated into envelope/stroma (ES) and thylakoid (Thy) fractions for immunoblot analysis. Rbcl and Lhcb1 were markers for stroma and Thy fractions, respectively. Each lane contained 5 μg of protein.

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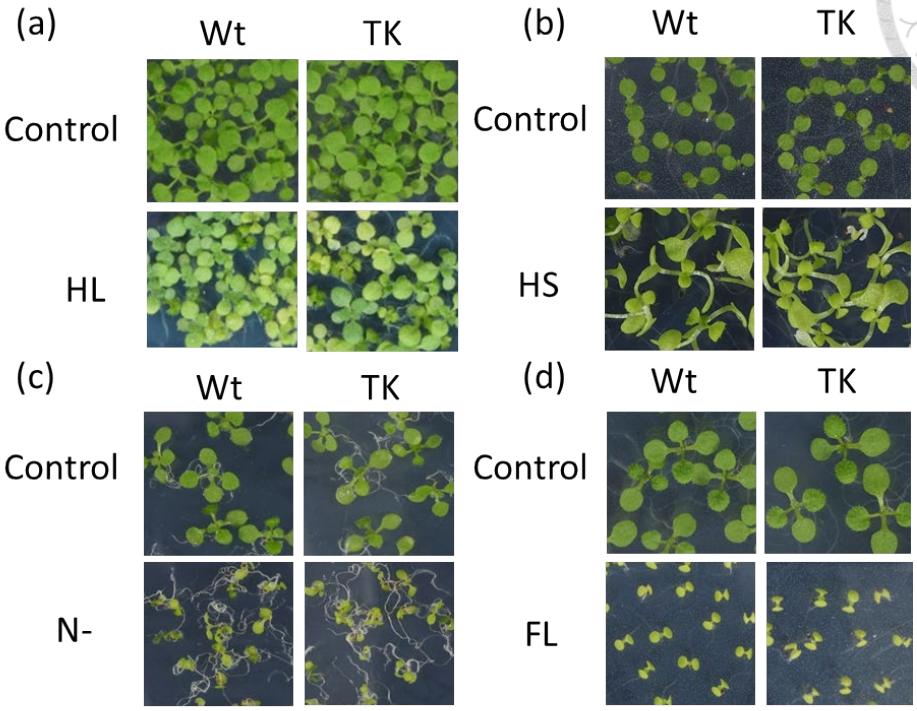

(e)

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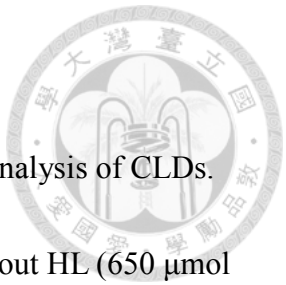

Figure 2. Seedlings grew under four stress conditions for functional analysis of CLDs. (a) , (d) Phenotypes of Wt and TK seedlings grew under with or without HL (650 μmol photons m^{-2} s^{-1}) and FL (10 μmol photons m^{-2} s^{-1} for 1 min, and 650 μmol photons m^{-2} s^{-1} for 5 min in turns during light periods) conditions for 11 d . (b) Phenotypes of 5-d-old Wt and TK seedlings before and after the HS (35/33°C, 16/8 light-dark cycle) treatment. Lower pictures were obtained after 7 d recovery in normal growth condition (80 μmol photons m^{-2} s^{-1}, 22°C, 16/8 light-dark cycle). (c) Phenotype of 7 d treatment of nitrogen deficiency in 3-d-old Wt and TK by shifting the plants to the 1/2 MS medium absent of nitrogen and sucrose. Control groups were grown on the1/2 MS medium without sucrose. (e) Appearance of 4-5 weeks old Wt and TK plants.

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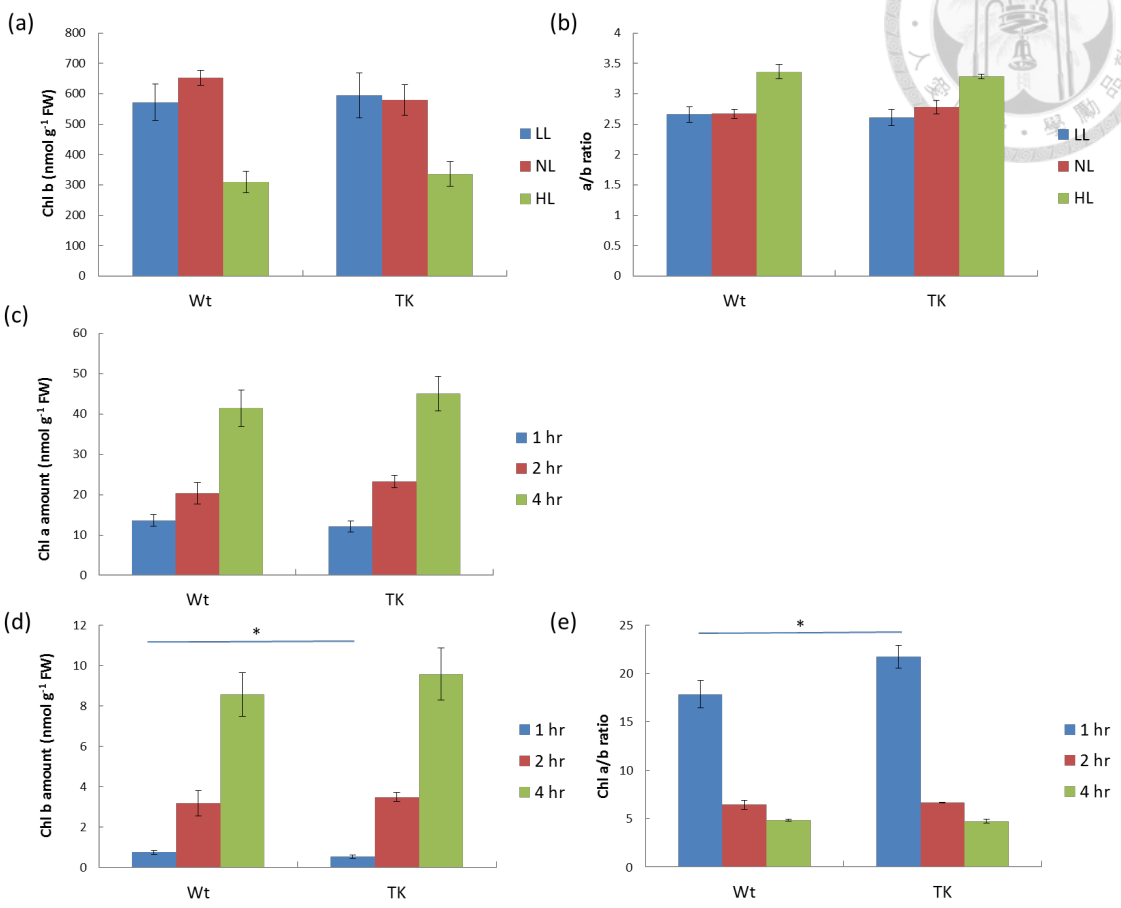

Figure 3. Chls content during light adaptation.

(a)-(b) Chls were extracted from 11-d-old seedlings grown under different light conditions (LL, 30 μmol photons m^{-2} s^{-1}; NL, 80 μmol photons m^{-2} s^{-1}; HL, 650 μmol photons m^{-2} s^{-1}). (c)-(e) 4-d-old etiolated seedlings were exposed to the LL condition and measured the Chls content and Chl *a*/*b* ratio at different timepoint. Data were averaged and analyzed with two-tailed t-test, and significant value were represented in * $P < 0.05$, error bars were SD (n = 3).

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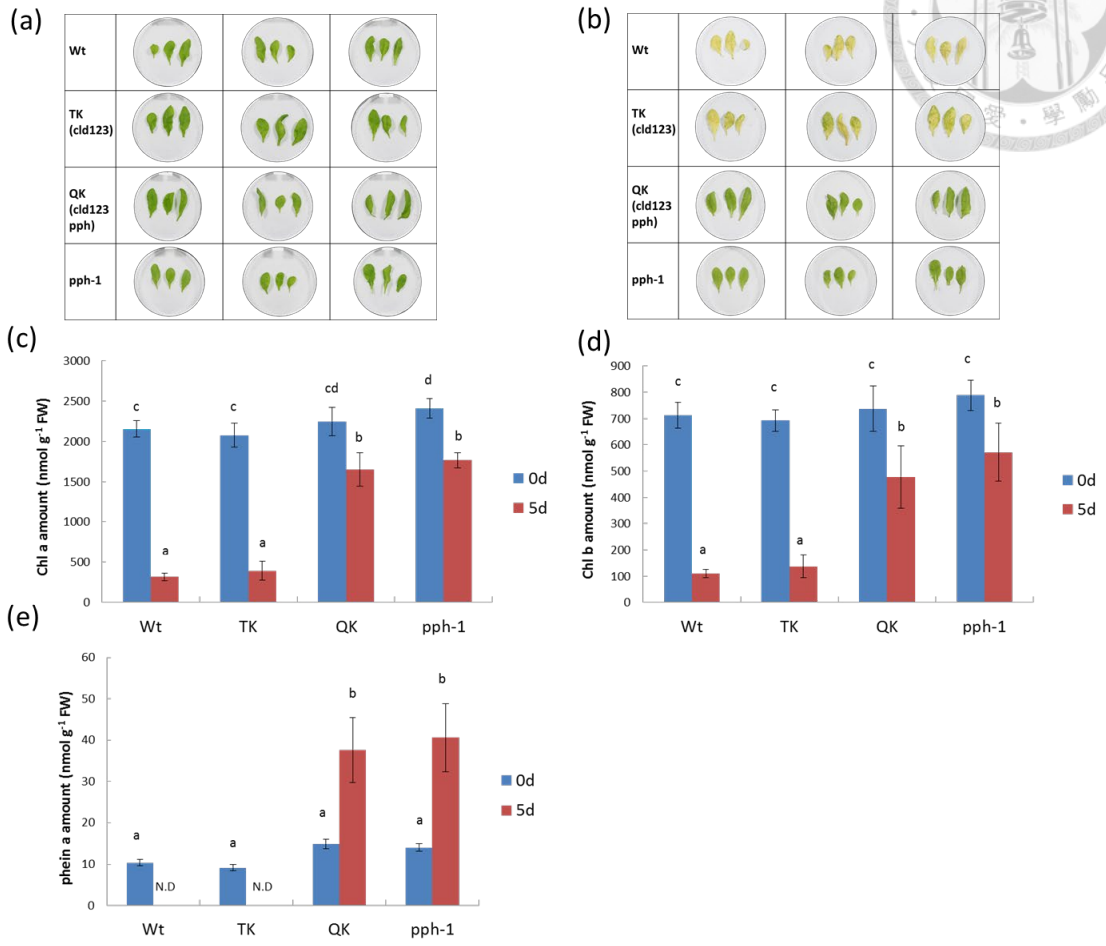

Figure 4. Appearance and Chls were measured after DILS (dark induce leaves senescence) treatment.

(a)-(b) Phenotypes of DILS are observed before and after the 5 d dark incubation. (c)-(e) Chls and Phein a were compared before and after DILS. Data were averaged and analyzed with two-way ANOVA with Duncan's method, significant value significant value in $P < 0.05$ are classified as a, b, c, d, error bars were SD (n = 3-8).

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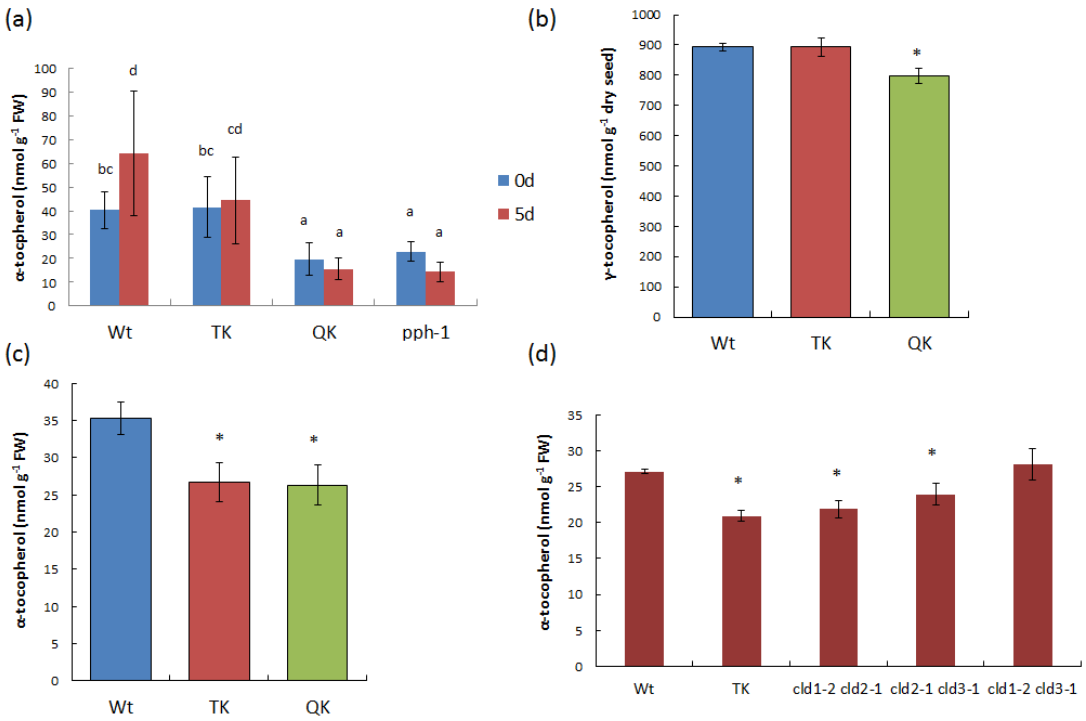

Figure 5. Analysis of tocopherol contents in mutants of Chl dephytylases.

(a) α-tocopherol contents before and after DILS treatment. (b) Different isoforms of tocopherol were analyzed in mature seeds. (c) 11-d-old seedlings without any treatment. (d) 11-d-old CLDs DK seedlings without any treatment were used for the determination of α-tocopherol amount. Wt and TK served as negative and positive control, respectively. Data were averaged and analyzed with two-way ANOVA with Duncan's method and two-tailed t-test, significant value in $P < 0.05$ are classified as a, b, c, d or *, error bars were SD (n = 3-8).

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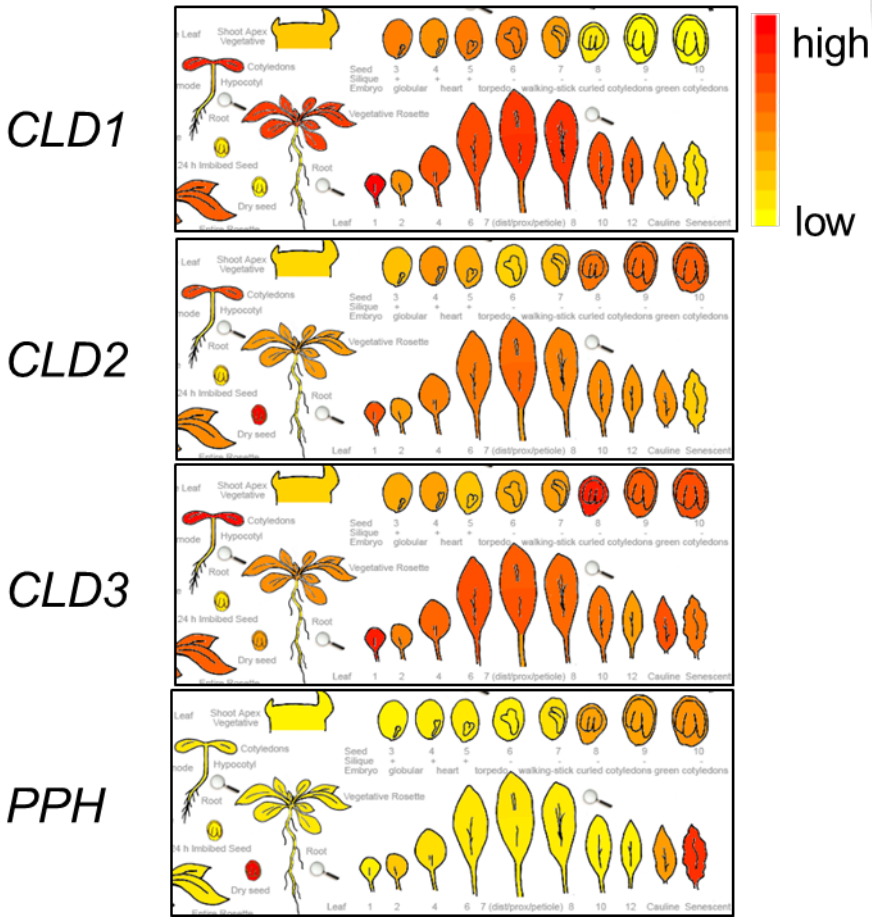

Figure 6. Arabidopsis gene expression profiles of CLDs and PPH in eFP browser format.

The expression profiles of *CLD1*, *CLD2*, *CLD3*, *PPH* are from the developmental map of Arabidopsis eFP Browser. Transcript levels are shown in yellow to red colors.

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Tables

Table 1. PCR reaction setting

(a) CLD1 wild-type primer

Cycle number	Denture	Anneal	Extend	Hold
1	94°C, 3 min			
2-31	94°C, 1 min	70°C, 1 min	72°C, 1 min	
32			72°C, 3 min	
33				20°C

(b) CLD1 mutant primer

Cycle number	Denture	Anneal	Extend	Hold
1	94°C, 3 min			
2-31	94°C, 1 min	68°C, 1 min	72°C, 1 min	
32			72°C, 3 min	
33				20°C

(c) CLD2, CLD3, PPH primer

Cycle number	Denture	Anneal	Extend	Hold
1	95°C, 3 min			

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2-4	95°C, 1 min	65°C, 1 min	72°C, 1 min 20 sec	
5-7	95°C, 1 min	60°C, 1 min	72°C, 1 min 20 sec	
8-34	95°C, 1 min	55°C, 1 min	72°C, 1 min 20 sec	
35			72°C, 3 min	
36				20°C

Table 2. Primer list for mutants genotyping

#	Name	Primer list	Sequence (5'-3')
1	CLD1-2	AtDh3-6F	ATGAGAGCTCTAACATGGACGGC
2	cld1-2	AtCLD1-2-R	ATAGCTAAAACCAGGTGGGC
3	CLD1-2	AtCLD1-2-wtR	GGTATAGCTAAAACCAGGTGGCT
4	cld2-1	AtAP22-LP	GTGTCAATGCATTCATGTTGC
5	cld2-1	AtAP22-RP	GAACTAGAGGCGAGCCTTCTC
6	cld2-1	LBb1.3	ATTTTGCCGATTTCGGAAC
7	cld3-1	AtPPH2-5F	CTGAAGTTCCAACGCTTTCAG
8	cld3-1	AtPPH2-5R	CACCTGTGACTGTTCATGCAC
9	cld3-1	Lb1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC

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10	pph-1	000095-F	CTACCAATCCTGGACTCCTCC
11	pph-1	000095-R	TGTACAGGTTATCGGTGAGCC
12	pph-1	LBb1.3	ATTTTGCCGATTTCGGAAC

Supplementary figure

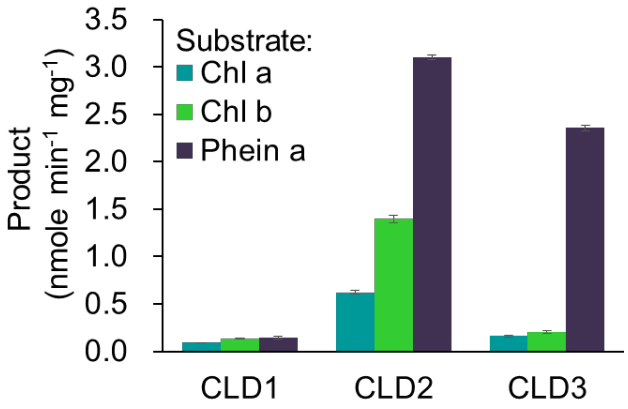

Figure 1. Dephytylation activity of CLDs with different substrates

Recombinant Arabidopsis $(His)_6$-CLDs were produced in *E. coli*. Activity assays were done by incubating the recombinant enzymes with 25 μM substrate for 30 min at 35°C, respectively. The products of dephytylation were analyzed by HPLC as previously described (Lin et al. 2016) and organized as a single figure.

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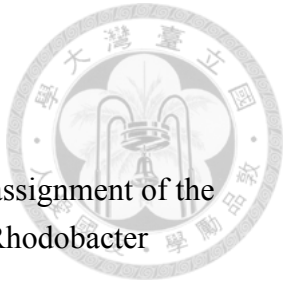

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