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Physiological Characteristics and Leaf Ultrastructure of a Novel Chlorophyll-deficient *chd6* Mutant of *Vitis venifera* Cultured in vitro

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Abstract A novel chlorophyll-deficient *chd6* mutant of F_1 hybrids from *Vitis venifera* was selected to study its primarily physiological characteristics and leaf ultrastructures under culturing in vitro. The results showed that although increasing Fe^{2+} and Mg^{2+} concentration could improve growth of the mutant in vitro, the effect was limited. In addition, it was determined that relatively lower Fe^{2+} and Mg^{2+} concentrations would be beneficial to the survival in vitro on GS medium. Chlorophyll contents of the mutant were significantly lower than those of its parents (4.53–13.76% of those of the higher-value parent Red globe). The chlorophyll *a/b* ratio was greatly increased (up to 4.34) to approximately twofold greater than that of its parents. Some critically successive enzymes for converting ALA to chlorophyll *a* could be inhibited to a variable extent in the *chd6* mutant, and more serious inhibition could happen in critical enzymes converting Mg-proto to Pchlide or Mg-proto to chlorophyll *b*. The mutant also showed not only poor Rubisco activities, lower percentage of dry matter, and soluble carbohydrate content, but also lower IAA and GA ($GA_1 + GA_4$) content and higher ABA content. In leaf ultrastructures, the mutant presented larger stomata size, higher percentages of stomata opening, and lower stomata density with more stoma approximately a ring shape. Most chloroplasts of the *chd6* mutant developed as asymmetric ellipses with deficient and irregular lamella.

Keywords *Vitis venifera* · Chlorophyll-deficient mutant · Culture in vitro · Chlorophyll · Rubisco activity · Soluble carbohydrates · Endogenous hormones · Chlorophyll synthesis precursors · Leaf ultrastructure

Introduction

Chlorophylls and their derivatives play a fundamental role in the energy absorption and transduction activities of all photosynthetic organisms. However, the chlorophyll-deficit mutation usually results in a depletion or even loss of photosynthetic pigments, resulting in an abnormal photosynthesis process. Morphologically, chlorophyll-deficient mutants are commonly characterized by visible mutation types such as albino, xanthan, virids, striata, tigrina, chlorina, zebra, and virescent to green-yellow, accompanied by dwarf and frail growth (Awan and others 1980; van Harten 1998; Huang and others 2005). In most higher plants, a chlorophyll mutation would be lethal, especially in albino mutants, or would result in variable plant ontogenesis (He and others 2006).

So far, chlorophyll-deficient mutants have been reported in many mono- or dicotyledonous plants due to spontaneous mutation (Lin and others 2003; Xu and others 2006), or chemical mutagenesis (Awan and others 1980; Zhao and others 2000; Tomoko and others 2002; Shibata and others 2004; Al-Qurainy and Khan 2009), T-DNA insertion (Jung and others 2003), and gene silencing (Kumar and Soll 2000; Monde and others 2000), in plants such as tobacco (Specht and others 1987), *Arabidopsis* (Runge and others

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1995; Lokstein and others 2002; Qin and others 2007; Kim and others 2009), tomato (Terry and Kendrick 1999), carrot (Nothnagel and Straka 2003), pea (Sokolskaya and others 2003), sunflower (Fambrini and others 2004), wheat (Falbel and others 1996; Wang and others 1996), maize (Greene and others 1998), barley (Bossmann and others 1999), and rice (Tomoko and others 2002; Jung and others 2003; Wu and others 2007; Huang and others 2008; Xu and others 2006; Chen and others 2007). Although numerous chlorophyll mutants would be considered a nonsense mutation due to the abnormal development or the early lethality in natural environments, more recent reports indicate that chlorophyll-deficient mutants have been used widely to study chloroplast/thylakoid structures and development (Bossmann and others 1999; Qin and others 2007; Kim and others 2009), photosynthesis of higher plants (Specht and others 1987; Lokstein and others 2002; Fambrini and others 2004), biosynthesis and metabolism of chlorophyll (Runge and others 1995; Qin and others 2007; Wu and others 2007), genetic differentiation (Tomoko and others 2002; Huang and others 2008), function identification and interaction of photosynthetic genes (Lopez-Juez and others 1998; Hansson and others 1999; Jung and others 2003; Huang and others 2005; Yu and others 2005; Qin and others 2007), and environment regulations, for example, light-induced morphogenesis (Parks and Quail 1991). In addition, chlorophyll mutants may also serve as a marker character in utilization of heterosis (Huang and others 2008).

Currently, although numerous mutants with alterations in chlorophyll synthesis have been identified in different plant species, to our knowledge there have been no previous reports of chlorophyll-deficient mutants that could dissect the chlorophyll biosynthetic pathway in detail, because they have pleiotropic effects on chloroplast structure and composition (Runge and others 1995). The pleiotropic nature of the majority of pigment mutations may have two possible explanations. First, mutations that disrupt any of the metabolic activities can potentially lead to a loss of chlorophyll, along with other plastid components, as a result of photooxidative damage (Somerville 1986). Second, accumulation of chlorophyll and development of the organelle appear to be interdependent and chlorophyll deficiencies may therefore be the result of defects in plastid development (Mascia 1978). Because of these pleiotropic effects, it has proven difficult in the past to clearly distinguish mutants with blocks in chlorophyll biosynthesis from those defective in other chloroplast activities (Runge and others 1995). Therefore, exploiting chlorophyll-deficient mutants from different plant species would be an efficacious approach to systematically study complicated problems associated with the chlorophyll biosynthetic pathway. Fortunately, we successfully obtained a new chlorophyll-deficient *chd6* mutant of *Vitis venifera* by a traditional sexual cross with two diploid cultivars, Phenix 51 and Red globe, combined by immature embryo culture of in vitro of F_1 hybrids. Test-tube plantlets of the chlorophyll-deficient *chd6* mutant cultured from 2001 to 2010 were quite consistent in phenotype: frail, nearly albino plantlets with small spots of green leaf color. The *chd6* mutant is interesting experimentally because no previous reports of chlorophyll-deficient mutants have been described in *Vitis venifera*. In the present study, physiological characteristics, including test-tube plantlet growth in different media, leaf chlorophyll and carotenoid content, leaf-soluble carbohydrate content, average percentage of dry matter (for example, average percentage of dry weight to fresh weight of single plantlet) of single plantlets, leaf ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) activity, leaf endogenous hormones, leaf chlorophyll synthesis precursor content, and leaf stomata and chloroplast ultrastructure, are characterized in a chlorophyll-deficient *chd6* mutant and its parents cultured in vitro.

Materials and Methods

Obtaining a New Chlorophyll-Deficient *chd6* Mutant of *Vitis venifera*

In 2001, by conducting the sexual cross of the two diploid cultivars Phenix 51 (the female parent with small berries and early maturation in the middle of August in Lanzhou, Gansu, China) and Red globe (the male parent with large berries and late maturation in early October in Lanzhou, Gansu, China), a total of 89 immature seeds of the F_1 hybrid were obtained 50 days after the first pollination. Among these 89 F_1 hybrid seeds, 53 immature seeds germinated to seedlings by the immature embryo culture method described in our previous report (Yang and others 2007), 41 of which were normal in phenotype (green color) and 12 of which were abnormal (chlorotic with approximate albino). Only one F_1 hybrid survived: the chlorophyll-deficient mutant named *chd6*. The other 11 abnormal plants died after about 20 days of culture. Because of the frail growth and low transplant survival of test-tube-grown *chd6* albino mutant plantlets with small spots of leaf color (Figs. 1 and 2), we conserved the special germplasm by subculturing in vitro in GS medium (Cao and Liu 2002) from 2001 to 2010.

Selection of Media and Culturing Condition

Since obtaining the *chd6* mutant in 2001, we tried to understand whether increasing Fe^{2+} and Mg^{2+} concentration could improve the growth in vitro of the *chd6* mutant. Based on the other components of the GS medium staying

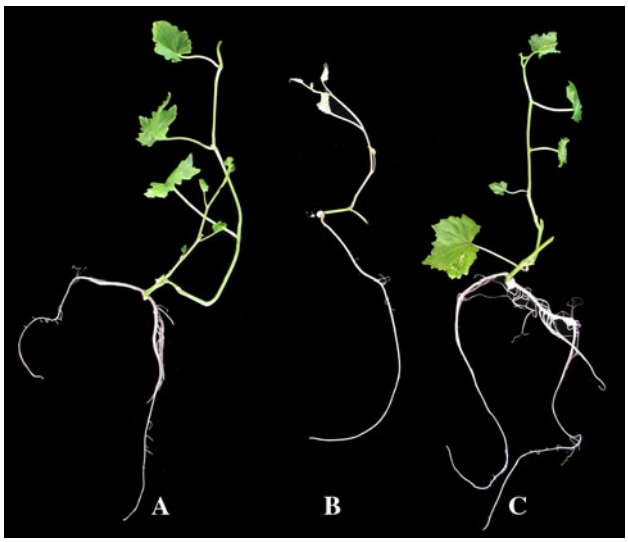

Fig. 1 Plantlets of Red globe (**a**), *chd6* mutant (**b**), and Phenix 51 (**c**) cultured in vitro after 30 days

invariable, the following GS medium designs were determined by GS-1: 6.95 mg l^{-1} $FeSO_4 \cdot 7H_2O$ + 62.5 mg l^{-1} $MgSO_4 \cdot 7H_2O$; GS-2: 13.9 mg l^{-1} $FeSO_4 \cdot 7H_2O$ + 125 mg l^{-1} $MgSO_4 \cdot 7H_2O$, which was Fe^{2+} and Mg^{2+} standard concentration of the GS medium (Cao and Liu 2002); GS-3: 27.8 mg l^{-1} $FeSO_4 \cdot 7H_2O$ + 250 mg l^{-1} $MgSO_4 \cdot 7H_2O$; GS-4: 41.7 mg l^{-1} $FeSO_4 \cdot 7H_2O$ + 375 mg l^{-1} $MgSO_4 \cdot 7H_2O$. All media were supplemented with 4.0 g l^{-1} agar, 30 g l^{-1} sucrose, and 0.1 mg l^{-1} indole-3-acetic acid (IAA). All media were adjusted to pH 5.8 with 0.1 M NaOH or 0.1 M HCl before autoclaving. Experiments were arranged in a factorial design with 20 conical flasks per treatment with three replications. Three stem segments (with a single shoot) were cultured per conical flask (50 ml), containing 25 ml of medium under fluorescent light (35–40 μmol m^{-2} s^{-1}) with 16 h photoperiod at 25 ± 2°C. After culturing in vitro for 30 days, the average proliferation multiple and average plantlet (with shoot and root) percentage of the *chd6* mutant and its parents were determined according to different media.

Examination of Physiological Traits

After in vitro subculturing of the *chd6* mutant and its parents on the GS medium for 30 days, leaf sampling was undertaken to determine chlorophyll and carotenoid contents, soluble carbohydrate contents, average percentage of dry matter (for example, percentage of dry weight to fresh weight) of single plantlets, total ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) activity, endogenous IAA, gibberellin (GA) (GA_1 + GA_4) and abscisic acid (ABA) contents, and content of the chlorophyll synthesis precursors [5-aminolevulinic acid (ALA); porphobilinogen (PBG); uroporphyrinogen III (Urogen III); coproporphyrinogen III (Coprogen III); protoporphyrin IX (Proto IX); Mg-protoporphyrin IX (Mg-proto); and protochlorophyllide (Pchlide)] of the *chd6* mutant and its parents for each sample, with three replications by the following methods, respectively. Chlorophyll and carotenoid contents were estimated as described by Zou (2000). Chlorophyll from leaves was extracted with 80% acetone and evaluated by a TU-1810 UV-visible spectrophotometer. Leaf soluble carbohydrates were extracted according to a modified procedure described by Wardlaw and Willenbrink (1994). Total amounts of leaf soluble carbohydrates were determined as fructose equivalents using the anthrone colorimetric assay (Yemm and Willis 1954) at 620 nm using a TU-1810 UV-visible spectrophotometer. Total activities of Rubisco of leaves were analyzed spectrophotometrically at 25°C according to Ward and Keys (1989). Extraction, purification, and examination of IAA, GAs (GA_1 + GA_4), and ABA were carried out using the method of the indirect enzyme-linked immunosorbent assay (ELISA) technique by an Multiskan-MK3 enzyme-labeled meter (Thermo Scientific), using the immunoassay detection kits described by Yang and others (2001). The chlorophyll synthesis

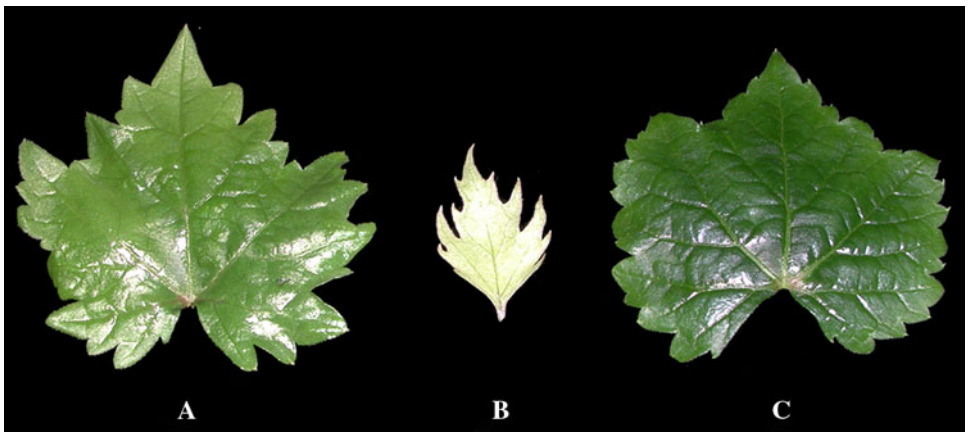

Fig. 2 Leaves of Red globe (**a**), *chd6* mutant (**b**), and Phenix 51 (**c**) cultured in vitro after 30 days

precursor ALA was extracted as described by Dei (1985). Isolation of PBG and Urogen III was performed using the method of Bogorad (1962); Coprogen III, Proto IX, Mg-proto, and Pchlide were determined according to the method of Rebeiz and others (1975).

Observation of Leaf Stomata and Chloroplast Ultrastructure

Samples of middle leaves of the *chd6* mutant and its parents were prepared for observing leaf stomata and chloroplast ultrastructure after 30 days of in vitro culture. To examine the chloroplast ultrastructure of the *chd6* mutant and its parents, leaves were processed by the method described by Teng and others (2006) and viewed with a JEM-1230 transmission electron microscope (JEOL Ltd, Tokyo, Japan). Thirty cells of the palisade parenchyma were observed to evaluate chloroplast size and profile, number of chloroplasts per cell, number of starch grains per chloroplast profile, number of osmisophilic granules per chloroplast profile, and the structure distribution of thylakoid membranes. Leaf stomata and the abaxial epidermis were characterized according to the method of Blanke and Belcher (1989) using a JSM-680LA scanning electron microscope (JEOL Ltd). Thirty images of the leaf stomata of the abaxial epidermis were used to quantify stomata density, size, and opening percentage of the *chd6* mutant and its parents.

Statistical Analysis

Analyses of variance (ANOVA) of the data were subjected to one-way analysis and *F*-test using SPSS ver. 10.0 (SPSS Inc., Chicago, IL, USA). Data analysis of multiple comparisons was performed using Duncan's new multiple-range method.

Results

In vitro Growth Differences of the *chd6* Mutant and its Parents in Different Media

After obtaining the *chd6* mutant, we found that the mutant grew more slowly and weakly in vitro with severe chlorosis than normal plantlets. Consequently, it was considered whether increasing Fe^{2+} and Mg^{2+} concentration could improve the growth in vitro of the *chd6* mutant. Under the gradient changes of halving (GS-1 medium), doubling (GS-3 medium), and tripling (GS-4 medium) concentrations of Fe^{2+} and Mg^{2+} compared to their standard (GS-1 medium) concentrations, the average proliferation multiple and the average plantlet (with shoot and root) percentage of test-tube plantlets showed significant differences ($P < 0.01$) between the *chd6* mutant and its parents (Figs. 3, 4). Generally, the growth in vitro of Phenix 51 and Red globe was better than that of the *chd6* mutant in different media. In two extreme concentrations of Fe^{2+} and Mg^{2+} of GS medium, no plantlet of the *chd6* mutant survived, but plantlets of its parents still had lower proliferation multiples (from 1.43 to 2.15) and lower plantlet percentages (from 5.33 to 18.67%). The highest proliferation multiple and plantlet percentage of test-tube plantlets of the *chd6* mutant were found in the standard concentrations of Fe^{2+} and Mg^{2+} of GS-2 medium, up to 3.21 and 33.84%, respectively; however, its parents showed the highest values in the GS-3 medium, up to 5.36 and 90.33% (Phenix 51) and 6.05 and 94.67% (Red globe), respectively. These results indicated that although increasing Fe^{2+} and Mg^{2+} concentrations could improve the growth in vitro of the *chd6* mutant, the effect was limited. Relatively lower concentrations of Fe^{2+} and Mg^{2+} could be beneficial to the survival in vitro on the GS medium.

Pigments of the *chd6* Mutant and its Parents

Chlorosis was a critical symptom for numerous chlorophyll-deficient mutants of higher plants (van Harten 1998; Huang and others 2005). To characterize the *chd6* mutant, we estimated the contents of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid in leaves of test-tube plantlets of the *chd6* mutant and its parents (Table 1). The pigment contents of the *chd6* mutant were significantly lower than those of its parents (4.53–13.76%; $P < 0.01$) compared to those of the higher-value parent Red globe.

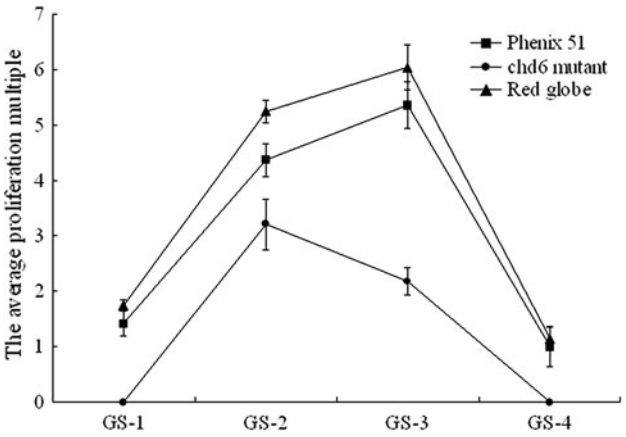

Fig. 3 The average proliferation multiple of the *chd6* mutant and its parents cultured in vitro in different media. GS-1: GS + 6.95 mg l^{-1} $FeSO_4 \cdot 7H_2O$ + 62.5 mg l^{-1} $MgSO_4 \cdot 7H_2O$; GS-2: GS + 13.9 mg l^{-1} $FeSO_4 \cdot 7H_2O$ + 125 mg l^{-1} $MgSO_4 \cdot 7H_2O$; GS-3: GS + 27.8 mg l^{-1} $FeSO_4 \cdot 7H_2O$ + 250 mg l^{-1} $MgSO_4 \cdot 7H_2O$; GS-4: GS + 41.7 mg l^{-1} $FeSO_4 \cdot 7H_2O$ + 375 mg l^{-1} $MgSO_4 \cdot 7H_2O$. All media were supplemented with 4.0 g l^{-1} agar, 30 g l^{-1} sucrose, and 0.1 mg l^{-1} indole-3-acetic acid

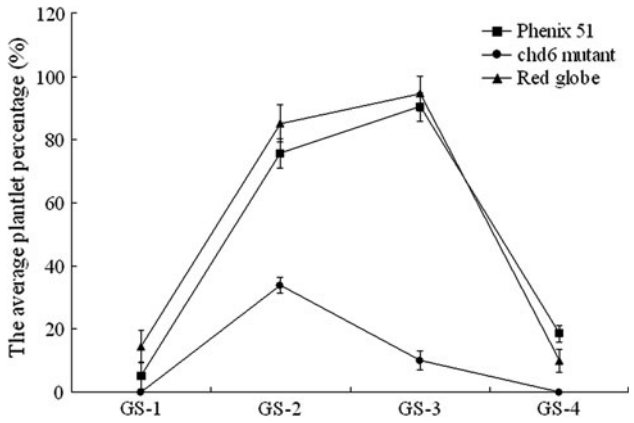

Fig. 4 The average plantlet percentage (%) of the *chd6* mutant and its parents cultured in vitro in different media. GS-1: GS + 6.95 mg l^{-1} $FeSO_4 \cdot 7H_2O$ + 62.5 mg l^{-1} $MgSO_4 \cdot 7H_2O$; GS-2: GS + 13.9 mg l^{-1} $FeSO_4 \cdot 7H_2O$ + 125 mg l^{-1} $MgSO_4 \cdot 7H_2O$; GS-3: GS + 27.8 mg l^{-1} $FeSO_4 \cdot 7H_2O$ + 250 mg l^{-1} $MgSO_4 \cdot 7H_2O$; GS-4: GS + 41.7 mg l^{-1} $FeSO_4 \cdot 7H_2O$ + 375 mg l^{-1} $MgSO_4 \cdot 7H_2O$. All media were supplemented with 4.0 g l^{-1} agar, 30 g l^{-1} sucrose, and 0.1 mg l^{-1} indole-3-acetic acid

The chlorophyll/carotenoid ratio of the *chd6* mutant was also drastically depleted. The chlorophyll *a/b* ratio of the *chd6* mutant, however, was greatly increased (up to 4.34), approximately twofold greater than that of its parents. The results indicated that the photosynthetic pigments of the *chd6* mutant had been seriously disorganized or depressed. Moreover, pigment changes adequately explain why the leaves of test-tube plants of the *chd6* mutant always present as approximate albino with small spot-green leaf color (Figs. 1, 2) at the level of the differences of the pigment content.

Comparison of Chlorophyll Synthesis Precursors Between the *chd6* Mutant and its Parents

To understand the potential of the enzyme system for converting ALA to chlorophyll *a*, it is important to evaluate the precursors of chlorophyll *a*. As shown in Fig. 5, relative contents (%) of PBG, Urogen III, Coprogen III, Proto IX, Mg-proto, and Pchlide were significantly lower in the *chd6* mutant than in its parents ($P < 0.05$). However, compared to other precursors of the *chd6* mutant, the relative content of Pchlide showed a drastic reduction, similar to levels of chlorophyll *a* or chlorophyll *b*. This result indicated that in the whole chlorophyll synthesis pathway, some critically successive enzymes could be inhibited to a variable extent in the *chd6* mutant, and the greatest inhibition might be in the conversion of Mg-proto to Pchlide or Mg-proto to chlorophyll *b*.

Average Dry Matter Percentage, Soluble Carbohydrate Content, and Rubisco Activity of the *chd6* Mutant and its Parents

As shown in Table 2, the *chd6* mutant possessed not only significantly lower dry matter percentage and soluble carbohydrate content, but also a lower Rubisco activity. Compared to the male parent Red globe, phenotypic values of three traits of the *chd6* mutant were decreased 70.68, 89.73, and 78.09% in turn. Correspondingly, the morphology of test-tube plantlets of the *chd6* mutant was characterized by slow growth, dwarf stature, and small leaves during subculturing; in particular, the upper stems became translucent or transparent when subcultured for over 25 days. These results indicate that the *chd6* mutant could be deficient in carbon dioxide fixation and remobilization and in accumulation of soluble carbohydrates during its growth and development.

Endogenous IAA, GA (GA_1 + GA_4), and ABA Content of the *chd6* Mutant and its Parents

Plant hormones play important roles in regulating the ontogeny of higher plants. To understand the differences of

Table 1 Differences of chlorophyll and carotenoid contents between the *chd6* mutant and its parents

Content (μg g^{-1} FW)	Phenix 51	*chd6* mutant	Red globe	*F* value
Chlorophyll *a*	$371.50^{b} \pm 11.63$ (65.97)	$53.81^{c} \pm 4.25$ (9.56)	$563.09^{a} \pm 16.67$ (100)	1381.00**
Chlorophyll *b*	$175.41^{b} \pm 7.31$ (64.08)	$12.41^{c} \pm 2.09$ (4.53)	$273.72^{a} \pm 10.45$ (100)	938.74**
Total chlorophyll	$546.91^{b} \pm 18.94$ (65.36)	$66.22^{c} \pm 6.34$ (7.91)	$836.81^{a} \pm 27.12$ (100)	1201.83**
Carotenoid	$52.83^{b} \pm 5.53$ (74.88)	$9.71^{c} \pm 1.24$ (13.76)	$70.55^{a} \pm 3.05$ (100)	212.75**
Chlorophyll *a/b*	$2.12^{b} \pm 0.02$ (102.91)	$4.34^{a} \pm 0.40$ (210.68)	$2.06^{b} \pm 0.02$ (100)	97.16**
Chlorophyll/carotenoid ratio	$10.40^{b} \pm 0.73$ (87.69)	$6.84^{c} \pm 0.22$ (57.67)	$11.86^{a} \pm 0.13$ (100)	100.15**

Values are $\mu \pm$ SD (%). The percent value of the *chd6* mutant and female parent was based on the assumption that the pigment content of the male parent Red globe was 100%

FW fresh weight of leaves, *SD* standard deviation

Data analysis in the same row of multiple comparisons was done with Duncan's new multiple-range method. The different lowercase letters showed significant difference at the level of 5%

**Showed very significant difference of the analysis of variance at $P \leq 0.01$. (%)

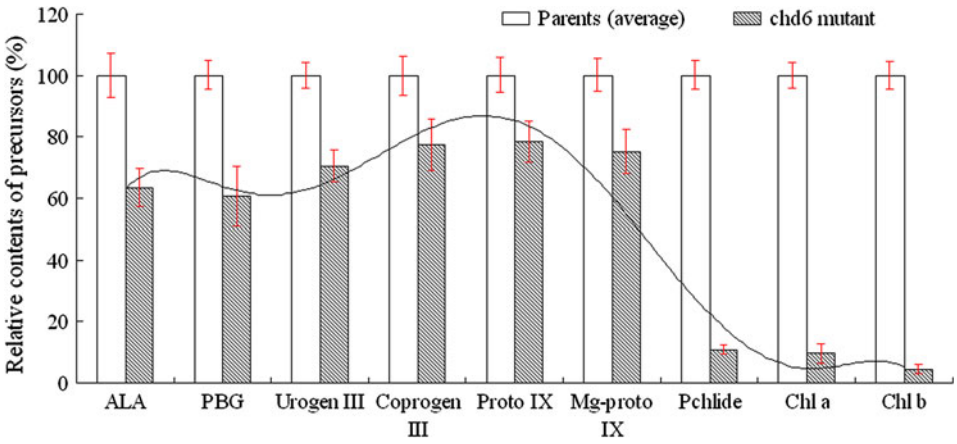

Fig. 5 Comparison of chlorophyll synthesis precursors between the *chd6* mutant and its parents (given that every precursor's content of the average of Phenix 51 and Hongti was 100% and compared to that of the *chd6* mutant)

Table 2 Differences of soluble carbohydrate content, the average dry matter percentage, and Rubisco activity between the *chd6* mutant and its parents

	Phenix 51	*chd6* mutant	Red globe	*F* value
Average dry matter percentage (%)	$12.61^{ab} \pm 1.02$ (65.68)	$5.63^{b} \pm 0.95$ (29.32)	$19.20^{a} \pm 0.97$ (100)	3.64*
Soluble carbohydrate content (μg g^{-1} FW)	$63.69^{b} \pm 3.75$ (88.53)	$7.46^{c} \pm 0.82$ (10.37)	$71.94^{a} \pm 1.53$ (100)	648.95**
Total Rubisco activities	$18.67^{b} \pm 1.24$ (85.06)	$4.81^{c} \pm 0.96$ (21.91)	$21.95^{a} \pm 1.38$ (100)	81.34**

Values are $\mu \pm$ SD (%). The percent value of the *chd6* mutant and female parent was based on the assumption that the average dry matter percentage, the soluble carbohydrate content, and total Rubisco activities of the male parent Red globe was 100%

FW fresh weight of leaves, *Rubisco* ribulose-1,5-biphosphate carboxylase/ oxygenase

The average dry matter percentage (%) was the percentage of dry weight to fresh weight of a single plantlet. Data analysis in the same row of multiple comparisons was performed using Duncan's new multiple-range method. The different lowercase letters showed significant difference at the level of 5%

*, ** showed very significant difference of the analysis of variance at $P \leq 0.05$ and $P \leq 0.01$, respectively

endogenous hormone levels between the *chd6* mutant and parents, we examined endogenous IAA, GA ($GA_1 + GA_4$), and ABA contents (Table 3). There was a significant decrease in IAA and GA content in leaves of the *chd6* mutant compared with those of its parents. Only 1.36 ng g^{-1} FW of IAA and 12.25 ng g^{-1} FW of GAs, which were approximately 10.92 and 9.31% of that of Red globe, were found in the *chd6* mutant. On the other hand, the ABA content of the *chd6* mutant was over threefold higher than that of its parents, up to 89.82 ng g^{-1} FW. Lower levels of IAA and GAs and higher levels ABA may explain the poor growth of the *chd6* mutant.

Leaf Stomata of the Abaxial Epidermis of the *chd6* Mutant and its Parents

Based on observations of leaf stomata size, opening percentage, and density on the abaxial epidermis, we also found significant differences ($P < 0.01$) between the *chd6* mutant and its parents (Table 4, Fig. 6). The *chd6* mutant stomata were longer (30.65 μm) and wider (29.69 μm), had a greater opening percentage (91.33%), and a lower density (205.83 stomata per mm^2) than its parents (Fig. 6a–c). Moreover, the stoma of the *chd6* mutant was more ring shaped. It was illustrated that although the mutation could increase stomata size of the abaxial epidermis, it was possibly at the expense of decreasing stomata density. On the other hand, stomata of the *chd6* mutant could be more deprived of the function of autoregulating stomata opening and closing compared to its parents.

Chloroplast Ultrastructure of the *chd6* Mutant and its Parents

When compared to its parents, the *chd6* mutant had no significant differences in chloroplast size (length and width) and number of chloroplasts per cell by analysis of variance ($P > 0.05$) (Table 5). However, noticeable differences were observed in chloroplast profile, number of starch grains and osmisophilic granules per chloroplast profile, and structure of thylakoid membranes between the *chd6* mutant and its parents. Chloroplasts were asymmetric ellipses in the *chd6* mutant (Fig. 7b), whereas most of the chloroplasts were oblate shapes in the parents (Fig. 7a, c).

Table 3 Endogenous GA_S ($GA_1 + GA_4$), IAA, and ABA content of the *chd6* mutant and its parents

	Phenix 51	*chd6* mutant	Red globe	*F* value
IAA (ng g^{-1} FW)	$10.97^a \pm 1.04$ (88.11)	$1.36^b \pm 0.38$ (10.92)	$12.45^a \pm 1.29$ (100)	5.05*
GA_S (ng g^{-1} FW)	$126.53^a \pm 14.71$ (96.21)	$12.25^b \pm 2.33$ (9.31)	$131.52^a \pm 11.57$ (100)	5.62*
ABA (ng g^{-1} FW)	$26.27^b \pm 2.81$ (107.53)	$89.82^a \pm 3.05$ (367.66)	$24.43^b \pm 2.33$ (100)	4.22*

Values are $\mu \pm$ SD (%). The percent value of the *chd6* mutant and the female parent was based on the assumption that the endogenous indole-3-acetic acid (IAA), gibberellins (GA_S ($GA_1 + GA_4$), and abscisic acid (ABA) contents of male parent Red globe was 100%

FW fresh weight of leaves, *SD* standard deviation

Data analysis in the same row of multiple comparisons was performed using Duncan's new multiple-range method. The different lowercase letters showed significant difference at the level of 5%

* showed very significant difference of the analysis of variance at $P \leq 0.05$

Table 4 Differences of stomata size and density of test-tube plantlets between the *chd6* mutant and its parents

	Phenix 51	*chd6* mutant	Red globe	*F* value
Stomata length (μm)	$26.13^c \pm 0.39$	$30.65^a \pm 0.12$	$27.41^b \pm 0.27$	204.05**
Stomata width (μm)	$22.52^b \pm 0.48$	$29.69^a \pm 0.63$	$21.81^c \pm 0.34$	172.49**
Stomata ratio length to width	$1.16^b \pm 0.01$	$1.03^c \pm 0.02$	$1.26^a \pm 0.03$	90.39**
Stomata opening percentage (%)	$77.16^b \pm 0.46$	$91.33^a \pm 0.21$	$67.49^c \pm 0.71$	1703.03**
Stomata density/mm^2	$388.54^a \pm 2.79$	$205.83^c \pm 1.53$	$341.38^b \pm 3.16$	4026.30**

Values are $\mu \pm$ SD

Data analysis in the same row of multiple comparisons was performed using Duncan's new multiple-range method. The different lowercase letters showed significant difference at the level of 5%

** showed very significant difference of the analysis of variance at $P \leq 0.01$

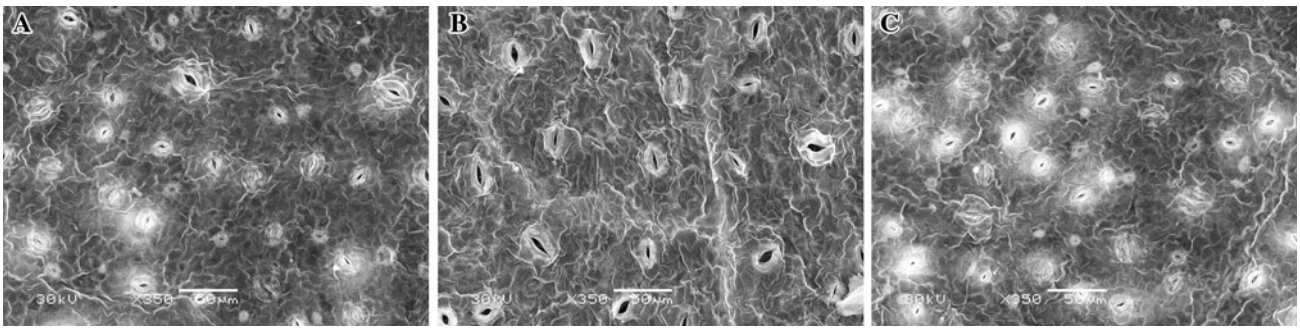

Fig. 6 Scanning electronic micrographs showing stomata structure of the abaxial epidermis of test-tube plantlet leaves of Phenix 51 (**a**), the *chd6* mutant (**b**), and Red globe (**c**)

Most of thylakoid lamellar membranes in the *chd6* mutant chloroplasts were irregular with no clear lamella and no complicated thylakoid system (Fig. 7b), similar to thylakoid membranes of etioplasts. No starch grains were found in the *chd6* mutant chloroplasts compared to the average one to three starch grains per chloroplast in the parents. On the other hand, more osmophilic granules were observed in the *chd6* mutant chloroplasts, up to 25.33 osmophilic granules per chloroplast profile on average, than in its parents (Fig. 7a–c, Table 5). These results indicate that the chloroplasts of the *chd6* mutant are abnormal, especially the thylakoid lamellar membrane system.

Discussion

Numerous chlorophyll-deficient mutants of higher plants have been isolated by the selection of spontaneous mutants (Lin and others 2003; Xu and others 2006) or by artificial mutagenesis (Awan and others 1980; Kumar and Soll 2000;

Table 5 Differences of chloroplast ultrastructure between the *chd6* mutant and its parents

	Phenix 51	*chd6* mutant	Red globe	*F* value
Chloroplast profile	Oblate	Asymmetric ellipse	Oblate	–
Chloroplast length (μm)	$2.40^{a} \pm 0.37$	$1.15^{a} \pm 0.32$	$2.06^{a} \pm 0.43$	2.38
Chloroplast width (μm)	$0.62^{a} \pm 0.19$	$0.75^{a} \pm 0.11$	$0.85^{a} \pm 0.16$	0.83
Number of chloroplasts per cell	$7.47^{a} \pm 0.83$	$5.84^{a} \pm 0.27$	$8.79^{a} \pm 0.52$	0.80
Number of starch grains per chloroplast profile	$1.55^{a} \pm 0.91$	0^{b}	$1.82^{a} \pm 0.53$	3.63*
Number of osmisophilic granules per chloroplast profile	$1.67^{b} \pm 0.87$	$25.33^{a} \pm 3.45$	$2.36^{b} \pm 1.21$	8.41**
Structure of thylakoid membranes	Abundant and regular	Deficient and irregular	Abundant and regular	–

Values are $\mu \pm$ SD

Data analysis in the same row of multiple comparisons was performed using Duncan's new multiple-range method. The different lowercase letters showed significant difference at the level of 5%

*, ** showed very significant difference of the analysis of variance at $P \leq 0.05$ and $P \leq 0.01$, respectively. *F* value with no asterisk meant difference of the analysis of variance at $P > 0.05$

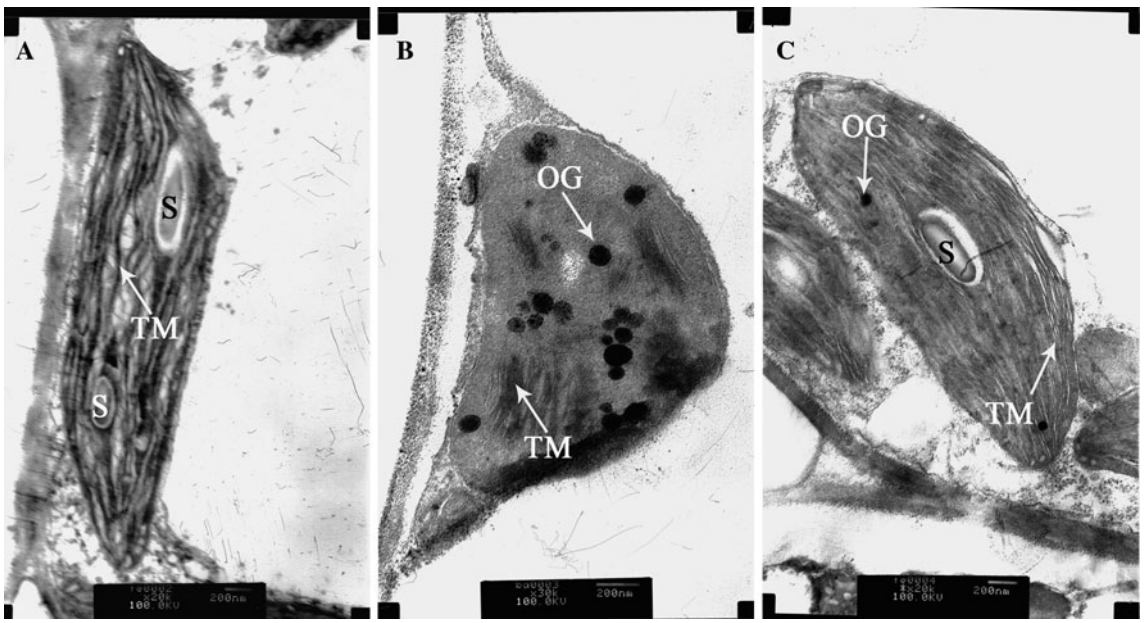

Fig. 7 Transmission electronic micrographs showing chloroplast ultrastructure of test-tube plantlet leaves of Phenix 51 (**a**), the *chd6* mutant (**b**), and Red globe (**c**). *S* starch grain, *TM* thylakoid membrane, *OG* osmisophilic granule

Monde and others 2000; Zhao and others 2000; Tomoko and others 2002; Jung and others 2003; Shibata and others 2004; Al-Qurainy and Khan 2009).These mutants are valuable for obtaining more information about the physiology and biochemistry of the metabolic pathways and the genetic mechanisms of mutants defective in chloroplast structure, function, and pigment synthesis (Runge and others 1995; Bossmann and others 1999; Tomoko and others 2002; Qin and others 2007; Wu and others 2007; Huang and others 2008; Kim and others 2009). However, as a result of the nature of the pleiotropic effect (Runge and others 1995) in different chlorophyll-deficient mutants and the complexity of chlorophyll metabolism of higher plants, isolating more chlorophyll-deficient mutants would be beneficial to the systematic study of the complicated process of chlorophyll biosynthesis. In our previous study, we obtained a new chlorophyll-deficient *chd6* mutant of *Vitis venifera* by the sexual cross (cv. Phenix 51 × cv. Red globe), combining the embryo culture in vitro. Moreover, we found that among the total of 53 F_1 progenies that survived, the ratio of normal progenies (green color) to abnormal progenies (chlorotic with approximate albino) was 3.42:1. This ratio is similar to the 3:1 ratio that is characteristic of a recessive nuclear gene, suggesting that the chlorophyll-deficient *chd6* mutant could be controlled by a recessive nuclear gene. The genetic phenomenon of the chlorophyll-deficient mutation was also found in barley (Liu and others 2008). Through observations of the

chlorophyll-deficient *chd6* mutant for about 10 years, the mutant steadily presented approximate albino with small spot-green leaf color and frail growth (Figs. 1, 2). Because of its low transplant survival rate, we have successfully established a method of conserving the *chd6* mutant in vitro. The mutant could be novel in *Vitis venifera*, and it was favorable for exploring the nature of the mutant involved in chlorophyll synthesis, as well as even improving the photosynthetic capabilities for grapevines.

Magnesium (Mg) and Iron (Fe) are very important constituents involved in plant growth and development, and especially required for chlorophyll biosynthesis of higher plants. Plants suffering from Mg and Fe deficiency always show decreased growth potential and a drastic reduction in some physiological functions (Cao and Tibbits 1992; Guller and Krucka 1993). Lu and others (1995) found that deficiencies of Fe and Mg decreased the amount of chlorophyll and protein but dramatically increased the amount of carotenoid in the chlorophyll-deficient mutant of *Arabidopsis thaliana*. Under field conditions, the symptom could be alleviated by Fe and Mg supplementation (Sanz and others 1997; Fernández and others 2008; Peng and others 2008). However, by estimating the average proliferation multiple and the average plantlet percentage of test-tube plantlets of the mutant and its parents in our experiment, although increasing Fe^{2+} and Mg^{2+} concentration could improve the growth in vitro of the *chd6* mutant, we found that the effect was limited compared to its parents. Relatively lower concentrations of Fe^{2+} and Mg^{2+} (13.9 mg l^{-1} $FeSO_4{\cdot}7H_2O$ + 125 mg l^{-1} $MgSO_4{\cdot}7H_2O$) would be beneficial to the survival in vitro of the mutant on GS medium, whereas parent plantlets were still capable of growing under extreme concentrations of Fe^{2+} and Mg^{2+} (GS-1: 6.95 mg l^{-1} $FeSO_4{\cdot}7H_2O$ + 62.5 mg l^{-1} $MgSO_4{\cdot}7H_2O$ and GS-4: 41.7 mg l^{-1} $FeSO_4{\cdot}7H_2O$ + 375 mg l^{-1} $MgSO_4{\cdot}7H_2O$) (Figs. 3, 4). This phenomenon indicated that the *chd6* mutant could be less efficient in absorbing, remobilizing, and metabolizing magnesium and iron.

Sivanesan and others (2008) stated that the reduction of chlorophyll content of in vitro cultures would reduce photosynthesis by decreasing light absorption. Thus, plantlets with higher chlorophyll content might have a greater chance of survival and better growth and development during acclimatization due to photosynthetic competence. However, the chlorophyll content of chlorophyll-deficient mutants was always depleted, resulting in a Chl *a/b* of more than the value of approximately 3 for normal plants (Havaux and Tardy 1997; Xu and others 2006; Chen and others 2007). In our study, the pigment contents of the *chd6* mutant were significantly depleted, being only 4.53–13.76% of that of the parent Red globe. The chlorophyll/carotenoid ratio of the *chd6* mutant was also drastically depleted, whereas the Chl *a/b* ratio of the *chd6* mutant was greatly increased up to 4.34, approximately twofold greater than that of its parents. Similarly, a novel rice mutant (*Oryza sativa* L. var. Zhenhui 249Y) was identified as a low chlorophyll *b* mutant with a Chl *a/b* ratio of 4.7, demonstrating that the mutant noticeably reduced PSII thermostability by the chlorophyll fluorescence assay (Chen and others 2007). Xu and others (2006) also found these results in the chlorophyll-deficient mutant *W1* of rice, which had a significantly lower amount of chlorophyll with a Chl *a/b* ratio of 5.0, and so did Light-Harvesting-Complex II (LHCII) trimers; Western blotting further revealed that LHCII apoproteins in *W1* dropped to one third of its amount in wild type. This phenomenon was interpreted to mean that mutants with a greater Chl *a/b* ratio inhibited LHCII assembly severely and thus decreased light-harvesting efficiency as well as PSII stability (Havaux and Tardy 1997). Of course, the chlorophyll physiology in higher plants is a complex and coordinated process that is executed via a series of cooperative reactions catalyzed by numerous enzymes (Beale 1999). Accordingly, we also found a significantly lower percentage of dry matter and soluble carbohydrate content, lower Rubisco activities, and decreased growth potential in the *chd6* mutant, indicating that the mutant phenotype could be the result of severe blockage of carbon dioxide fixation and the remobilization and accumulation of soluble carbohydrates during its growth and development, which could be one of the reasons why the *chd6* mutant grew slowly and weakly with severe chlorosis.

Chlorophyll is assembled in chloroplasts from eight molecules of ALA by a network of pathways leading to the formation of various tetrapyrroles (Eckhardt and others 2004), and the main next-to-last precursors in chlorophyll synthesis in angiosperms are followed as PBG, Urogen III, Coprogen III, Proto IX, Mg-proto IX, Pchlide, and Chlide (Wettstein and others 1995). If one step of chlorophyll biosynthesis is blocked, the former precursors should be bypassed and chlorophyll precursors accumulated, and the latter precursors should decrease. Mascia (1978) clearly demonstrated that in normal maize plants this results in a buildup of protoporphyrin IX and protochlorophyllide, while mutants defective in chlorophyll synthesis accumulate precursors, depending on the site of the mutant-induced lesion. In the chlorophyll-less barley mutant *NYB*, the chlorophyll biosynthesis mutation can be attributed to a blockage in the conversion of Pchlide to Chl *a*, indicating that the enzymes catalyzing Pchlide were most likely damaged (Liu and others 2008). However, we found more complicated results, suggesting that the accumulation of chlorophyll biosynthetic precursors (from ALA to Pchlide) in the *chd6* mutant was significantly lower than its parents' average (the control), opposite that found in previous studies (Mascia 1978; Wettstein and others 1995; Liu and

others 2008). The relative content of Pchlide was also much reduced in the *chd6* mutant, as was chlorophyll *a* or chlorophyll *b*, indicating that some critically successive enzymes could be inhibited to a variable extent in the *chd6* mutant in the whole chlorophyll synthesis pathway, and more serious inhibition could happen in critical enzymes converting Mg-proto to Pchlide or Mg-proto to chlorophyll *b*.

Plant hormones influence almost every aspect of the plant's life cycle (Sun and Gubler 2004). IAA and GA_3 could retard senescence (Davies 1987; Rosenvasser and others 2006; Li and others 2010) and delay chlorophyll degradation to maintain the chlorophyll content (Li and others 2010). By contrast, ABA could inhibit shoot growth and counteract the effect of IAA and GA_3 on promoting plant growth and development (Davies 1987). In the present study, there were lower levels of IAA (1.36 ng g^{-1} FW) and GAs ($GA_1 + GA_4$) (12.25 ng g^{-1} FW) and higher levels of ABA (89.82 ng g^{-1} FW) in leaves of the *chd6* mutant compared with those of its parents, which could be one of the important reasons why the *chd6* mutant grows slowly and poorly. Stomata are specialized cells that affect plant adaptation (Brownlee 2001) by directing transpiration and photosynthesis (Nabin and others 2000). Stomata number can be affected by factors such as genotype, phenophase, and growing location (Sabo and others 2001). Other research has indicated that stomata are stably inherited (Yang and others 1998), and some quantitative values such as stomata density, stomatal size, and stomatal index are very significant in differentiating the taxa at the species and interspecies level (Kliewer and others 1985; Caglar and Tekin 1999; Nabin and others 2000). In this study we also found similar results by microscopic observation of leaf stomata of the abaxial epidermis. The *chd6* mutant had larger stomata size, higher percentage of stomata opening, and lower stomata density compared to its parents. Moreover, the stoma of the *chd6* mutant was more ring shaped. These results indicated that the mutant stomata might not be functioning normally under in vitro culture conditions.

Chlorophyll-deficient mutants of higher plants generally have abnormal development in chloroplast ultrastructure, such as irregular shapes, disrupted thylakoid membranes, and undifferentiated proplastids (Jung and others 2003; Qin and others 2007). The present data demonstrate that most of chloroplasts of the *chd6* mutant developed as asymmetric ellipses with deficient and irregular lamella. However, there was no significant difference in chloroplast size and number of chloroplasts per cell between the *chd6* mutant and its parents. In a rice chlorophyll-deficient mutant, Jung and others (2003) showed that although no change in the number of chloroplasts was observed, the shape of thylakoid membranes in the *OschlH* mutant was irregular and severely disrupted compared with the wild-type chloroplasts. The reduction of light-harvesting complexes in the thylakoid membrane due to the lack of chlorophyll synthesis may disrupt the thylakoid ultrastructure in the mutant. Interestingly, we also found no starch grain accumulation in chloroplasts of the *chd6* mutant, which suggests that the mutant has lost the capacity for photosynthesis to a degree due to the anomalies of thylakoid lamellar membrane system.

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