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The title page**The title of article:**

Pigment comparison and expression of chlorophyll metabolism genes in yellow and green *Acer palmatum* leaves

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Abbreviations

Carotenoid Car

Chlorophyll Chl

Chlorophyll synthase CHLG

Chlorophyllase Chlase

Chlorophyllide Chlide

Chlorophyllide *a* oxygenase CAO

Green wild-type GW

NADPH:protochlorophyllide oxidoreductase POR

RNA-interference RNAi

Yellow mutant YM

Abstract Exterior leaf color is an important trait for ornamental foliage plants, but the molecular mechanism underlying the variation in color between yellow- and green-leaf *Acer palmatum* is poorly understood. Indeed, chlorophyll (Chl) contents and gene transcriptions were very different between the yellow mutant (YM) and green wild-type (GW) leaves of this species. Here, annual variations of leaf color and pigment contents in two varieties were investigated. Then, transcript levels of six genes involved in Chl metabolism were quantitatively measured using real-time PCR in YM and GW leaves. Compared with GW, the color changes of YM leaves were classified into four typical periods. During the yellow leaf stage, YM showed greatly reduced Chl synthesis and a higher carotenoid (Car)/Chl ratio than GW. These phenomena may be the main cause of yellow leaves in YM. In addition, expression levels of Chl synthesis genes (*ApPORA*, *ApPORB*, *ApCAO* and *ApCHLG*) in YM were very low during this period. Concomitantly, the amount of transcripts of genes for Chl degradation (*ApCLH1* and *ApCLH2*) rose significantly compared with GW. Once YM leaves turned red, the above differences between the two genotypes lessened. Overall, our results indicated that changes in Chl synthesis and degradation may play important roles leading to the yellow leaves of YM *A. palmatum*.

Keywords: Leaf color variation; Pigment; Gene expression; *Acer palmatum*

Introduction

Species of the *Acer* genus (*Aceraceae*) are famous for attractive leaf colors and shapes and, therefore, are important decorative trees throughout the world (Wada and Ribbens 1997). *Acer palmatum* Thunb. is widely cultivated in Chinese gardens and has evolved several morphologically diverse ecotypes, which exhibit various rates of adaptability and ornamental value (Copini et al. 2014; Li et al. 2011, 2015a, 2015b; Rong et al. 2015; Schmitzer et al. 2009a, 2009b; Wada and Ribbens 1997). A yellow *A. palmatum* mutant (YM) was bred with its main characteristic being leaves that are golden-yellow color during April–August, compared with the typical green leaves of the wild-type (GW) plants (Li et al. 2011). As predicted, less chlorophyll (Chl) was detected in YM leaves than in GW (Li et al. 2015a, 2015b). In addition, some differentially expressed genes were identified from transcriptome data (Li et al. 2015a). However, little effort has been made to elucidate the underlying mechanisms of the annual change in YM leaf color.

Chl is the main pigment in plant leaves. It determines leaf color and plays unique, essential roles in photosynthetic light-harvesting. Biochemical analyses of Chl metabolism can contribute to identifying its encoding genes. Biosynthesis of Chl is classified into three distinct phases (Eckhardt et al. 2004). In the late reaction, NADPH:protochlorophyllide oxidoreductase (POR) catalyzes conversion of protochlorophyllide to chlorophyllide (Chlide) *a*, which exists in light-dependent form in all photosynthetic organisms (Masuda and Takamiya 2004). Concomitantly, the interconversion of Chlide *a* and Chlide *b* occurs, forming a chlorophyll cycle, which is regulated by chlorophyllide *a* oxygenase (CAO) (Beale 1999; Tanaka and Tanaka 2011). Finally, chlorophyll synthase (CHLG) esterifies Chlide and so Chl formation is accomplished (Eckhardt et al. 2004; Moulin and Smith 2005; Tanaka and Tanaka 2007; Willows 2003). Previously, chlorophyllase (Chlase) was known as the first and main enzyme for chlorophyll degradation, which removes hydrophobic phytol chains from Chl and forms Chlide (Barry 2009; Eckhardt et al. 2004; Harpaz-Saad et al. 2007; Hörtensteiner 2006; Takamiya et al. 2000). Several studies have confirmed that two isoforms (*CLH1* and *CLH2*) of the *Chlase* gene exist in many plants (Chen et al. 2014; Jacob-Wilk et al. 1999; Okazawa et al. 2006; Schenk et al. 2007; Tsuchiya et al. 1997). Despite the low homology of whole amino acid sequences among plant Chlases (Takamiya et al. 2000), their encoding genes play a distinctive role in Chl catabolism *in vivo* (Liao et al. 2007). However, the Chlase function in chlorophyll degradation was challenged (Schenk et al. 2007), and is not considered to be the main enzyme for Chl degradation during leaf senescence in *Arabidopsis*

thaliana (Schelbert et al. 2009).

Variations in color intensity can be attributed to differences in expression of Chl biosynthesis and degradation genes. For example, it is well known that multiple *POR* genes differ in their expression patterns. The *A. thaliana* *PORB* operates throughout the greening process and in light-adapted mature plants, while *PORA* is active in etiolated seedlings at the beginning of illumination (Masuda and Takamiya 2004). However, the *PORC* transcript was undetectable in etiolated seedlings, but increased after illumination (Oosawa et al. 2000; Su et al. 2001). It has been shown that the existence of differentially regulated *POR* genes is a general phenomenon among angiosperms (Masuda and Takamiya 2004). Further experiments showed that *porA-1* and *PORA* RNA-interference (RNAi) lines displayed Chl reduction (Paddock et al. 2012). More seriously, the *porB-1 porC-1* mutant displays a severe xantha (i.e. highly Chl-deficient) phenotype, which could be functionally rescued by addition of ectopically expressed *PORA* (Paddock et al. 2010). The transfer of *Arabidopsis* plants from moderate to shaded light intensity resulted in decreased *CAO* mRNA levels, accompanying a reduction in greenness of leaves, and vice versa (Harper et al. 2004; Tanaka and Tanaka 2005). Moreover, *CAO* over-expressing lines have remarkably changed Chl synthesis (Nakagawara et al. 2007; Pattanayak et al. 2005). Expression of *CHLG* is also involved in feedback-control of Chl biosynthesis. Similar to *Arabidopsis chlg* mutants, *NtCHLG* antisense transgenic tobacco plants exhibited a pale-green leaf phenotype with white/yellow-green variegated leaves (Lin et al. 2014; Shalygo et al. 2009). In the case of *CLHs*, they displayed varied transcriptional activity in different leaves of *Pachira macrocarpa* (Chen et al. 2014). Two *AtCLHs* exhibited differential expression patterns and the contents of Chlide *a* and *b* in leaves of their RNAi lines were substantially reduced (Liao et al. 2007). The evidence shown above indicates that leaf color is closely related to transcription of Chl metabolism genes, thus analysis of the expression pattern of these genes in *A. palmatum* will help to reveal the mechanism of the annual color change.

Although Chl presence in *A. palmatum* is important for its photosynthesis and leaf ornamental value, knowledge of the molecular genetics of Chl metabolism in this species is still sparse. Anthocyanins have been studied, but there has been little focus on Chl content in different colored leaves (Ji et al. 1992; Li et al. 2015a, 2015b; Schmitzer et al. 2009a, 2009b). Additionally, only partial sequence data from *A. palmatum* are available in GenBank. Fortunately, the transcriptome data from YM and GW could provide valuable information on sequence formation of Chl biosynthesis and

degradation (Li et al. 2015), which will be useful for research on molecular biological differences between YM and GW leaves. In this study, the annual variation of leaf color and pigment contents in YM and GW were investigated. To analyze *A. palmatum* leaf color variation at the molecular level, we tested expression profiles of six genes (*ApPORA*, *ApPORB*, *ApCHLG*, *ApCAO*, *ApCLH1* and *ApCLH2*) for Chl metabolism in *A. palmatum* plants of the two different colors during the growth season.

Materials and methods

Plant materials

Every 15 d, middle-positioned leaves growing on upper branches with the same direction of two *A. palmatum* (i.e. GW and YM) were collected from the Maple source nursery in Jiangsu Academy of Agricultural Sciences, Nanjing, China (32.040°N and 118.877°E) throughout the growing season of 2015. Leaves were collected from nine trees, with three leaves per tree, in each genotype at each time point and were classified randomly into three groups (class I, class II and class III) for taking photographs or extracting photosynthetic pigments or total RNA, respectively. Finally, the annual color changes of different leaves were measured based on the Royal Horticultural Society Colour Chart (Fifth Edition).

Pigment isolation and analysis

Leaf tissue (0.1 g) from each genotype was immersed in 10 ml of a 9:1 (v/v) mix of acetone:(0.1 M) NH_4OH to extract Chl *a*, Chl *b* and carotenoid (Car), which were measured according to Li et al. (2015a, 2015b). The content of each pigment was expressed as $\text{mg}\cdot\text{g}^{-1}$ FW. Each sample was tested three times, and averages were used as the final result for this measurement.

RNA extraction and quantitative real-time RT-PCR analyses (qPCR)

Total RNA was extracted from leaves in two groups using the RNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. At the same time, RNase free DNase I (Takara, Kyoto, Japan) was adding to avoid DNA contamination. Then, first-strand cDNA was synthesized using an M-MLV RTase cDNA Synthesis Kit (Takara). Specific primers for *ApPORA*, *ApPORB*, *ApCAO*, *ApCHLG*, *ApCLH1* and *ApCLH2* (Table 1) were designed based on the cDNA sequences from *A. palmatum* transcriptome information (Gene ID: Maple 24507, 25097, 29179, 34202, 22632 and 14335; Li et al. 2015). Then, qPCR was carried out to determine the expression levels of these genes at different growth times. The *ApEF-1a* gene was chosen as the internal control in this experiment and qPCR was run on a Thermal Cycler Dice Real Time System (TP800, Takara) using a previously

reported reaction program and conditions (Li et al. 2015a). Three independent biological replicates, each with two technical replicates, were analyzed. A no-template reaction was used as a negative control. The comparative Ct value was used to calculate the relative gene expression. The RNA level was expressed relative to the expression of *ApEF-1α* as $2^{-\Delta\Delta CT}$, according to previous analyses (Pfaffl 2001).

Results

Annual fluctuation of leaf color

The annual changes in leaf color of *A. palmatum* YM were investigated to better understand the leaf color feature. The annual variation in leaf color was clearly divided into four phases (Fig. 1): bud (27 March), yellow leaf (11 April to 24 August), faded yellow (8 September to 8 October) and turn red stages (23 October to 7 December). In early spring (27 May, bud stage), leaf buds from YM were red-group 43A, while GW buds were greyed-orange group 168B. After leaf emergence, the leaves of YM were typically yellow, and this color remained until the end of August (from yellow group 13B to 5D, yellow leaf stage); meanwhile, GW displayed green leaves (from yellow-green group 151C to green group 141C). In September, the yellow of YM leaves gradually faded (yellow 2C) and turned green (yellow-green group 144C). Consequently, they became green leaves with few and small yellow spots near the main veins by 8 October (green group 137D, faded yellow stage). However, the leaves of GW barely change color (from yellow-green 151C to green group 137C at the same time). After 23 October, the leaves of both YM (greyed-orange group 176D) and GW (grey-brown group 199B) began to turn red from the edges, and all leaves were completely red by 7 December (greyed-red 180B).

Pigment changes between two *A. palmatum* genotypes

Comparison of pigment contents in YM and GW leaves showed that the color variability reflected differences in Chl *a*, Chl *b* and Car concentrations and their ratios (Fig. 2). Our experiments revealed that four pigment (total Chl, Chl *a*, Chl *b* and Car) contents and Chl *a/b* in YM leaves and in GW leaves were similar at bud stage, while the Car/Chl in YM was significant higher than in GW at the same time.

During the yellow leaf stage, green pigment (total Chl, Chl *a* and Chl *b*) contents in YM leaves sharply decreased (as low as 3.91, 4.37 and 2.14 % of the GW control, respectively) (Fig. 2). This trend was closely negatively correlated with the development of leaf yellow coloration. It is very interesting that the synthesis of yellow pigment (Car) also reduced in YM and in GW leaves; however, the former

was more greatly reduced. However, the ratios of Chl *a/b* and Car/Chl in YM leaves were much higher than those in GW leaves during this period (at the peak reaching 2.52- and 15.63-fold of the GW, respectively).

In contrast to the Car/Chl values, the total Chl, Chl *a*, Chl *b* and Car contents were significantly lower in YM than in GW leaves at the faded yellow stage. In addition, there were slight differences in the Chl *a/b* ratios between the two varieties. When leaves began to turn red, the values of six pigment indexes (total Chl, Chl *a*, Chl *b* and Car contents, and ratios of Chl *a/b* and Car/Chl) converged in YM and GW leaves. This phenomenon was consistent with changes in their leaf colors during the turn red stage. The analysis in this study demonstrated that the color changes of the two genotypes were related to their Chl contents and Car/Chl ratio (Figs. 1 and 2a–c and f).

Gene expression differences

In conjunction with the observed differences in pigment contents between the two genotypes of *A. palmatum*, the transcript abundances of six genes (*ApPORA*, *ApPORB*, *ApCHLG*, *ApCAO*, *ApCLH1* and *ApCLH2*) involved in Chl metabolism were investigated using qPCR (Fig. 3). These six genes were expressed in YM leaves, with significant differences in their transcript levels in the GW plant.

Compared with GW, the expression levels of two *POR* genes (*ApPORA* and *ApPORB*) in YM leaves were reduced at the bud, yellow leaf and the initial part of faded yellow stages, with only 14.44%–93.45% of those in GW. After that time, their transcript amounts began to rise and even exceeded those in GW leaves by the end of the turn red stage, with the highest being 4.70- and 1.76-fold that of GW (Fig. 3a and b). However, expression of the *CAO* gene *ApCAO* was the opposite of the changes of Chl *a/b* values in both YM and GW leaves throughout the year. *ApCAO* transcript levels were much lower in YM than in GW, falling to 3.98 % of the latter (Fig 3c). In the case of Chl synthase, *ApCHLG* transcript abundance in YM was obviously lower than in GW, and was positively correlated with Chl content during the whole growing season (Figs. 2a and 3d). Different patterns were observed when the expressions of *ApCLH1* and *ApCLH2* were analyzed in YM and GW leaves, with *ApCLH2* having higher expression in both genotypes (Fig. 3e and f). *ApCLH1* exhibited one expression peak in YM leaves by 11 April (1.32-fold of that in GW), but its transcript level decreased and could not be detected when YM leaves began to turn red (23 October to 7 December). The *ApCLH2* expression intensities were always stronger in YM than in GW (Fig. 3f), and reached a maximum expression in YM leaves by 7 November (1.26-fold of that in GW). The range of results demonstrate

different expression patterns of the above common genes of Chl metabolism depending on varietal characteristics and plant developmental stage.

Discussion

Gene expression in *A. palmatum*

In most plants, Chl production is largely limited to leaves and occurs at different development stages. The visible accumulation of its compounds usually reflects the activity of enzymes functioning in the Chl biosynthetic pathway (Eckhardt et al. 2004). In *A. palmatum*, analysis of the transcriptional data revealed one or two expression peaks for each of the Chl biosynthetic genes *ApPORA*, *ApPORB*, *ApCAO* and *ApCHLG* during the growth season in the GW plants (Fig. 3a–d). A similar pattern of common gene transcript abundance peaking in early period or at the same time was also observed in YM leaves, but showed lower expression levels (Fig. 3a–d). Leaves are complex tissues in which Chl synthesis and degradation are simultaneously active throughout their entire life cycle (Barry 2009; Eckhardt et al. 2004; Harpaz-Saad et al. 2007; Hörtensteiner 2006; Moulin and Smith 2005; Takamiya et al. 2000; Tanaka and Tanaka 2007; Willows 2003). Some researchers considered that Chlase is the first rate-limiting enzyme for Chl breakdown, which removes the hydrophobic phytol chain from this pigment, hence converting Chl to Chlide (Eckhardt et al. 2004; Harpaz-Saad et al. 2007; Hörtensteiner 2006; Takamiya et al. 2000). However, the presence of one transcript abundance peak for *ApCLH1*/*ApCLH2* was not correlated with a gradual increase in Chl degradation either in YM or GW leaves from spring to autumn (Figs. 2a and 3e and f). This result indicated that *Chlase* may not play an important role in Chl degradation (Schenk et al. 2007; Schelbert et al. 2009). Overall, our results indicate that transcripts of *ApPORA*, *ApPORB*, *ApCAO* and *ApCHLG* were abundant, allowing synthesis of Chl in GW leaves in the growth season (Fig. 3). Nevertheless, there were different patterns in expressions of these six genes between YM and GW leaves.

Much evidence has shown that multiple *POR* genes differ in their expression pattern, but all function in Chl synthesis (Masuda and Takamiya 2004; Oosawa et al. 2000; Su et al. 2001). Either single or multiple *por* mutants of *Arabidopsis* invariably present different degrees of leaf color fading, even of xantha type (Paddock et al. 2010, 2012). In the present study, except for the turn red stage, the expression levels of *ApPORA* and *ApPORB* were higher in green leaves from GW than in yellow/yellow-green variegated leaves from YM (Fig. 3a and b), the profiles of which were consistent with Chl accumulation (Fig. 2a). A similar pattern was observed in mature leaves of cucumber (Kuroda

et al. 2000). Our data additionally revealed that *ApCAO* was expressed in leaf tissues of both *Acer* plants, and was closely related to the depth of green in leaves throughout the year (Figs. 1 and 3c). Uniform results were previously obtained in leaves of *Arabidopsis* (Harper et al. 2004; Nakagawara et al. 2007; Pattanayak et al. 2005; Tanaka and Tanaka 2005). In their studies, decreases in *CAO* mRNA levels were accompanied by a reduction in greenness of leaves, and vice versa. In addition, *CHLG*, as the last gene of the Chl biosynthetic pathway, tightly coordinates Chl synthesis, and its transcriptional level was positively correlated with Chl contents, ultimately affecting leaf color (Lin et al. 2014; Shalygo et al. 2009). The *ApCHLG* gene was expressed in both yellow and green *A. palmatum* leaves, while its expression was stronger in GW than in YM (Fig. 3d). A possible explanation of this transcript pattern may involve the consideration of many factors, such as the genetic background of the two plant variants.

Meanwhile, expression of *CLHs*, regardless of involvement in Chl degradation, also affect leaf Chl contents and color (Chen et al. 2014; Eckhardt et al. 2004; Harpaz-Saad et al. 2007; Hörtensteiner 2006; Liao et al. 2007; Takamiya et al. 2000). As shown in the present study, *CLHs* (*ApCLH1* and *ApCLH2*) each had one expression peak at 11 April or 7 November, respectively (Fig. 3e and f). The transcript levels of *CLHs* (*ApCLH1* and *ApCLH2*) were higher in YM yellow leaves than in GW green leaves, and were negatively related to the Chl content (Figs. 2a and 3e and f). These data indicate that the relationship between Chl biosynthesis and leaf color in *A. palmatum* may be controlled by complex regulatory mechanisms.

Coloring mechanism of YM leaf

The YM leaf color mutant is useful for investigating molecular mechanisms involved in the Chl metabolism and color control of *A. palmatum*. Mutations in structural genes in Chl biosynthesis or degradation can produce mutants with reduced or increased Chl contents (Harpaz-Saad et al. 2007; Liao et al. 2007; Lin et al. 2014; Paddock et al. 2010, 2012; Pattanayak et al. 2005; Shalygo et al. 2009; Tanaka and Tanaka 2005). Additionally, appropriate agronomic practices can regulate leaf color by affecting expression of these genes (Chen et al. 2014; Harper et al. 2004; Li et al. 2015b; Nakagawara et al. 2007; Pattanayak et al. 2005; Tanaka and Tanaka 2005). In the present study, we found that YM presented a quite different leaf color in yellow leaf (11 April to 24 August) and faded yellow stages (8 September to 8 October) compared with GW controls (Fig. 1). Therefore, the pigment contents and expression profiles of six genes for Chl metabolism in the two genotypes of *A. palmatum* were

investigated to reveal the YM coloring mechanism.

Once leaves emerged, YM entered the yellow leaf stage, with its leaves typically yellow compared to green leaves of GW (Fig. 1). As we expected, accumulation of green pigments (total Chl, Chl *a* and Chl *b*) were significantly lower in YM than in GW leaves (the former was just a few percent of the latter, Fig. 2a–c). These changes were negatively correlated with the degree of yellow coloration of YM leaves (Fig. 1). Indeed, Chl content directly influenced the leaf color, furthermore, pale green or etiolation genotypes usually have some defects in Chl biosynthesis (Harper et al. 2004; Li et al. 2015b; Paddock et al. 2010, 2012; Pattanayak et al. 2005; Shalygo et al. 2009; Tanaka and Tanaka 2005). Compared with GW, the transcripts of genes *ApPORA*, *ApPORB*, *ApCAO* and *ApCHLG* were scant in YM leaves, resulting in insufficient synthesis of Chl for green coloration (Figs. 2a and 3a–d). However, the transcript levels of glutamyl tRNA reductase (*HemA*) and Mg-chelatase subunit (*CHLH*, *CHLI* and *CHLD*) in both leaves did not show significant differences according their transcriptome data (Li et al., 2015a). It is a pity that more homology genes of *HemA* and *CHLI* were not identified and detected from *A. palmatum* until now. Some advanced technologies, such as the whole genome sequencing and single molecule real-time sequencing for transcriptome, will be help to complete understand the molecular regulatory mechanism of Chl biosynthesis in this species. Of course, the capacity for Chl breakdown is also an important factor affecting leaf color (Chen et al. 2014; Eckhardt et al. 2004; Harpaz-Saad et al. 2007; Hörtensteiner 2006; Liao et al. 2007; Lin et al. 2014; Shalygo et al. 2009; Takamiya et al. 2000). In our experiment, two *CLH* genes (*ApCLH1* and *ApCLH2*) were highly expressed in yellow leaves of YM, accompanying the sharp decline in Chl content (Figs. 2a and 3e and f). Overexpression of a heterologous *Chlase* gene was responsible for the chlorotic phenotype of squash plants (Harpaz-Saad et al. 2007). However, based on the fact that *Chlase* may not function in Chl degradation (Schenk et al. 2007; Schelbert et al. 2009), more experimental evidence is needed to explain the relationship between *CLH* expression and Chl content in *A. palmatum*. In summary, our results indicate that reduction in Chl synthesis may be the main reasons for yellow leaves in *A. palmatum*, a process regulated at transcription level.

After August, YM reached the faded yellow stage and the yellow of its leaves gradually faded and turned green, while GW leaves remained green (Fig. 1). Just as last phase, the contents of total Chl, Chl *a*, Chl *b* and Car were significantly lower in YM than in GW leaves (Fig. 2a–d). However, Chl pigments began to accumulate in YM leaves, and reached ~3.5 times those at the end of the yellow leaf

stage (Fig. 2a–d). The results were consistent with the changes in leaf color from yellow to green (Fig. 1). It was not surprising that expression levels of several Chl biosynthesis genes (*ApPORA*, *ApPORB*, *ApCAO* and *ApCHLG*) gradually increased simultaneously with the deepening green of leaves (Fig. 3a–d). As previously mentioned, these genes are involved in Chl synthesis and take part in regulation of leaf color (Harper et al. 2004; Kuroda et al. 2000; Lin et al. 2014; Masuda and Takamiya 2004; Nakagawara et al. 2007; Oosawa et al. 2000; Paddock et al. 2010, 2012; Pattanayak et al. 2005; Su et al. 2001; Shalygo et al. 2009; Tanaka and Tanaka 2005;). To some extent, leaf color control can be realized through adjusting their transcription. Our experiments showed that *A. palmatum* enhanced the expression of Chl biosynthesis genes when YM leaves changed from yellow to green. Among them, the amount of *ApCHLG* transcripts dramatically increased, reaching 8.24-fold of levels at the end of the yellow leaf stage (Fig. 2d). *CHLG* tightly coordinates Chl synthesis and effectively controls leaf color, and the transcriptional level was positively correlated with Chl contents (Fig. 2d; Lin et al. 2014; Shalygo et al. 2009). Thus, our results led to the hypothesis that the change from yellow to green in YM leaves of *A. palmatum* is mainly controlled by *CHLG* expression.

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Figure legend

Fig.1 The annual fluctuation of leaf color in green wild-type (GW, upper row) and yellow mutant (YM, lower row) of *Acer palmatum*. Eighteen sampling dates—Mar 27; Apr 11, Apr 26, May 11, May 26, Jun 10, Jun 25, Jul 10, Jul 25, Aug 9, Aug 24; Sep 8, Sep 23, Oct 8; Oct 23, Nov 7, Nov 22 and Dec 7 — are designated as bud stage, yellow leaf stage, fade yellow stage and turn red stage, respectively. The colorimetric measurement on leaves color was analyzed basing on the Royal Horticultural Society Colour Chart (Fifth Edition).

Fig. 2 The annual comparison of pigment contents in green wild-type (GW) and yellow mutant (YM) leaves of *Acer palmatum*. The amount of total chlorophyll (a), chlorophyll *a* (b), chlorophyll *b* (c) and carotenoids (d), the ratio of chlorophyll *a/b* (e) and carotenoids/chlorophyll (f) were showed as the means $\pm$ standard errors (*bars*) of three replicate experiments.

Fig. 3 Expression analysis of six genes involves in chlorophyll synthesis or degradation in green wild-type (GW) and yellow mutant (YM) leaves of *Acer palmatum*. The RNA extraction, cDNA acquisition and quantitative real-time PCR operations were carried out as described in the “Materials and methods” section. The transcript levels of *ApPORA* (a), *ApPORB* (b), *ApCAO* (c), *ApCHLG* (d), *ApCLH1* (e) and *ApCLH2* (f) were normalized to *ApEF-1 α* , which was amplified as an internal control, in the same samples. Values are expressed as means $\pm$ standard errors (*bars*) of three replicate experiments.

82x56mm (300 x 300 DPI)

Fig. 2

166x254mm (96 x 96 DPI)

Fig. 2

169x250mm (96 x 96 DPI)

170x247mm (96 x 96 DPI)

Fig. 3

170x253mm (96 x 96 DPI)

Table 1 Primers used for quantitative real-time RT-PCR analyses in *Acer palmatum*

Gene name	Primer name	Sequence (5'→3')	Length (bp)	Gene length (bp)	Position (mRNA)	Tm (°C)	GC %	Product length (bp)
<i>ApEF-1α*</i>	EF-1α-F	TCGTCCCCATCTCTGGTTTT	20	1121	569	61.79	50.00	91
	EF-1α-R	AGGGCATCAAGGAGGGTTG	19		659	62.36	57.89	
<i>ApPORA</i>	PORA-F	TGAATTCTCTCTTGCATCTCAACAC	25	1648	1317	61.98	40.00	148
	PORA-R	TTAGTTTCCTCTGCCTTCTCCATC	24		1464	62.18	45.83	
<i>ApPORB</i>	PORB-F	CTTCTTTTCCCACCCTTCCA	20	1727	1092	61.34	50.00	81
	PORB-R	CACCTGAGCCAGTCTTTGTCC	21		1172	62.18	57.14	
<i>ApCAO</i>	CAO-F	GAGGCAGTAACGAAGGAATGGTAG	24	1202	1013	62.42	50.00	147
	CAO-R	CAACAGATGGAAAATGCGAGAA	22		1159	62.37	40.91	
<i>ApCHLG</i>	CHLG-F	CCGAATGAGGGGAAAATGAGA	20	1612	1300	62.25	50.00	147
	CHLG-R	GGTGGGAAATGGGAGTGAAA	20		1446	62.02	50.00	
<i>ApCLH1</i>	CLH1-F	CACAGAACTCAATCCCAGCATC	22	1305	546	61.97	50.00	94
	CLH1-R	TTTTGCCACCCACCAA	17		639	61.94	52.94	
<i>ApCLH2</i>	CLH2-F	CTTGCTGGTCATAGTCGTGGAG	22	1423	580	62.13	54.55	136
	CLH2-R	GTGGTGGAGTTTGTTCCTTT	22		715	61.48	45.45	

* Elongation factor 1 alpha (*ApEF-1α*) served as the internal control.