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Transcriptome Analysis of a Subtropical Deciduous Tree: Autumn Leaf Senescence Gene Expression Profile of Formosan Gum

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Autumn leaf senescence is a spectacular natural phenomenon; however, the regulation networks controlling autumnal colors and the leaf senescence program remain largely unelucidated. Whether regulation of leaf senescence is similar in subtropical deciduous plants and temperate deciduous plants is also unknown. In this study, the gene expression of a subtropical deciduous tree, Formosan gum (*Liquidambar formosana* Hance), was profiled. The transcriptomes of April leaves (green leaves, 'G') and December leaves (red leaves, 'R') were investigated by next-generation gene sequencing. Out of 58,402 de novo assembled contigs, 32,637 were annotated as putative genes. Furthermore, the *L. formosana*-specific microarray designed based on total contigs was used to extend the observation period throughout the growing seasons of 2011–2013. Network analysis from the gene expression profile focused on the genes up-regulated when autumn leaf senescence occurred. *LfWRKY70*, *LfWRKY75*, *LfWRKY65*, *LfNAC1*, *LfSPL14*, *LfNAC100* and *LfMYB113* were shown to be key regulators of leaf senescnece, and the genes regulated by *LfWRKY75*, *LfNAC1* and *LfMYB113* are candidates to link chlorophyll degradation and anthocyanin biosynthesis to senescence. In summary, the gene expression profiles over the entire year of the developing leaf from subtropical deciduous trees were used for in silico analysis and the putative gene regulation in autumn coloration and leaf senescence is discussed in this study.

Keywords: Formosan gum • Leaf senescence • *Liquidambar formosana* • Microarray • Next-generation sequencing • Transcriptome.

Abbreviations: ACD, accelerated cell death; CAO, chlorophyllide *a* oxygenase; CCE, chlorophyll catabolic enzyme; C4H, cinnamate-4-hydroxylase; Chl, chlorophyll; ChlG, chlorophyll synthase; CHI, chalcone isomerase; CHS, chalcone synthase; 4CL, 4-coumaroyl:CoA-ligase; DFR, dihydroflavonol 4-reductase; DVR, divinyl chlorophyllide a 8-vinyl reductase; ERF, ethylene response factor; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′ hydroxylase; GO, gene ontology; GSA, glutamate-1-semialdehyde aminotransferase; HCAR, hydroxymethyl chlorophyll *a* reductase; LDOX, leucoanthocyanidin dioxygenase; LHCII, light-harvesting complex II; MBW complex, MYB–bHLH–WD repeat complex; NGS, next-generation sequencing; NYC1, NONYELLOW COLORING1; ORF, open reading frame; PAL, phenylalanine ammonia-lyase; PaO, pheophorbide *a* oxygenase; PAP, production of anthocyanin pigments; PORA, NADPH, protochlorophyllide oxidoreductase A; PORC, NADPH, protochlorophyllide oxidoreductase C; PPH, pheophytinase, pheophytin pheophorbide hydrolase; PPO, protoporphyrinogen IX oxidase; RACE, rapid amplification of cDNA ends; RNA-seq, whole-transcriptome sequencing; RCCR, red chlorophyll catabolite reductase; SAG, senescence-associated gene; SGR, SGN, STAYGREEN; SPL, squamosa promoter-binding-protein-like; UF3GT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase.

The raw transcriptome reads reported in this paper have been submitted to the NCBI Short Read Archive under accession Nos. SRR1514949 (green leaves of *L. formosana*, 'G') and SRR1514913 (red leaves of *L. formosana*, 'R'). The transcriptome contigs sequences are available in the ContigViews database (www.contigviews.bioagri.ntu.edu.tw)

Introduction

Leaf senescence is the final stage of leaf development in plants, and is tightly controlled and influenced by the environment (Lim et al. 2003). Autumnal leaf senescence may be particularly complex due to the remarkable coloration. As a conspicuous phenomenon marking the end of the growing season, the biology, ecology and physiology of autumn leaf senescence have been extensively studied (Matile 2000, Feild et al. 2001, Hoch et al. 2001, Keskitalo et al. 2005, Ougham et al. 2005, Schaberg et al. 2008). The two major coloration events of autumn leaf senescence are chlorophyll degradation and anthocyanin biosynthesis (Ougham et al. 2005), but the regulatory pathways connecting leaf senescence and coloration are largely unknown.

Chl degradation is visualized as loss of green color in leaves (Woo et al. 2013). It is the most obvious event of the onset of

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senescence, and so it is often used as a marker to determine the stages of leaf senescence (Buchanan-Wollaston 1997). The biosynthesis and degradation pathways of Chl have been almost fully characterized (Tanaka and Tanaka 2006, Hörtensteiner 2013). Chl degradation proceeds via the degradation of the Chl catabolite pheophorbide by pheophorbide *a* oxygenase (PaO) to form colorless linear tetrapyrroles (Pruzinská et al. 2003, Hörtensteiner 2013). The regulation of *PaO* is unknown, although it is specifically expressed when leaves start senescing. *STAYGREEN* (*SGR*, *SGN* or *NYE1*) was proposed to be a candidate regulator of *PaO*, but it was shown to be independent and to function upstream of *PaO* (Aubry et al. 2008).

The anthocyanin regulation network is in most part understood. Well-known anthocyanin regulators such as *PRODUCTION OF ANTHOCYANIN PIGMENTS 1* (*PAP1*/*AtMYB75*), *PAP2*/*AtMYB90* and *AtMYB113* in Col-0 wild-type Arabidopsis are members of the MYB–bHLH–WD repeat (MBW) complex (Gonzalez et al. 2008, Dubos et al. 2010). Recently, an explicit regulation network has been demonstrated in Petunia, marking out the regulatory roles of the MBW complex conserved across Rosids and Asterids (Albert et al. 2014). In perennials it was found that *MYB10* homologs in Rosaceae play an important role in the regulation of fruit anthocyanin accumulation and were associated with environmental factors such as temperature and light (Lin-Wang et al. 2011, Li et al. 2012). *MYB10* orthologous genes have been studied in perennial plants other than Rosaceae, but studies have mostly focused on fruit, with leaf senescence barely mentioned (Butelli et al. 2012).

Autumn leaf senescence and coloration have been mentioned in particular in studies of several temperate deciduous plants such as *Populus* spp., *Acer* spp., *Quercus* spp., *Betula* spp., *Fagus* spp. and *Ginkgo* spp. (Matile 2000, Schaberg et al. 2008, Xu et al. 2008, Kontunen-Soppela et al. 2010). In subtropical climates, many species grow at low altitude and exhibit autumn leaf senescence, including species of the genera *Acer* and *Liquidambar*. Formosan gum (*Liquidambar formosana* Hance.) is a perennial native of Taiwan. It is deciduous and in some habitats exhibits autumnal colored leaves for >3 months. Thus, it is a species well suited for studies of autumn leaf coloration of subtropical plants. Compared with temperate deciduous plants, studies on subtropical deciduous plants are rare.

Next-generation sequencing (NGS) has become a powerful tool for studies of non-model species (Neale and Kremer 2011). Along with whole-transcriptome sequencing (RNA-seq), it has enabled the monitoring of gene expression of species that have not hitherto been researched in molecular biology studies (Jain 2012). Perennials are characterized by large genomes and long generation times, thus complicating research efforts (Neale and Kremer 2011). To date, studies using NGS technology and bioinformatics have tended to focus on the many physiological aspects of woody plants, including development phase transition, hormonal state and phenotype studies (Krost et al. 2013, Mu et al. 2013, Qiu et al. 2013).

In order to investigate the regulation of autumn leaf senescence in Formosan gum, samples from green and red leaves were used to represent developing and senescing leaves, respectively. Genes were profiled by whole-transcriptome NGS, and a customized microarray was used to observe gene expression throughout the growing seasons. Classification and function prediction were carried out by ContigViews (Liu et al. 2014), and bioinformatics annotations were acquired. Network analyses were performed and a putative regulatory network was compiled, based on gene expression correlation. Finally, relationships in the network revealed a predicted link between leaf senescence and coloration, and candidate participating genes were identified.

Results

Leaf development and autumn senescence of Formosan gum

Formosan gum is a subtropical, relic deciduous species. The growing seasons of Formosan gum in northern Taiwan start from March. The leaves are fully expanded around July; coloration starts in November and ends with leaf fall in February the following year. The leaf color changes with the seasons (**Fig. 1A** and **B**, spring and winter, respectively). The sprout and senescing leaf showed pigment accumulation (**Fig. 1C**) which correlated with anthocyanin content (**Fig. 1D**). Chl content was also dynamic, increasing and reaching the highest accumulation around August, then decreasing gradually (**Fig. 1E**). As Chl content is often used as a metabolic marker of senescence (Sarwat et al. 2013), we concluded that in the Chl content observation year 2011–2013, Formosan gum started to senesce around August.

Transcriptome profiling and contig classification

Two individual libraries from developing and senescing leaves were constructed for deep sequencing to create the transcriptome profiles. A sample collected in April 2011 was used to represent developing leaves. This sample was named 'G' (green leaves). Based on the appearance and anthocyanin content, the sample collected in December 2013 was named 'R' (red leaves). The 58,402 contigs acquired using de novo assembly from both libraries are named 'LfGR contigs' (**Fig. 2**); the N_{50} is 1,214 nucleotides (**Supplementary Table S1**). After BLAST analysis by ContigViews (Liu et al. 2014), 32,637 of the contigs were annotated as putative genes. An open reading frame (ORF) prediction using ContigViews showed that there were 9,372 contigs with a full-length ORF (classified as 'complete') and 19,053 contigs with a partial ORF (classified as 'partial'). Another 4,151 contigs had sequences similar to the published sequences in TAIR or EMBL, but their ORFs were not well defined, and so were named 'others' (other than complete or partial genes, they may also be pseudogenes or sequencing errors). The remaining 25,765 contigs were named 'unannotated', and contributed about 44% of the transcriptome (**Supplementary Fig. S1A**), which may include non-coding RNA.

After BLAST, global alignment was performed using EMBOSS Needle (McWilliam et al. 2013). The best hits sequence according to the BLAST result was considered the reference contig. Of the complete contigs, about 2,500 of 9,372 contigs had nucleotide identities between 35% and 40% with

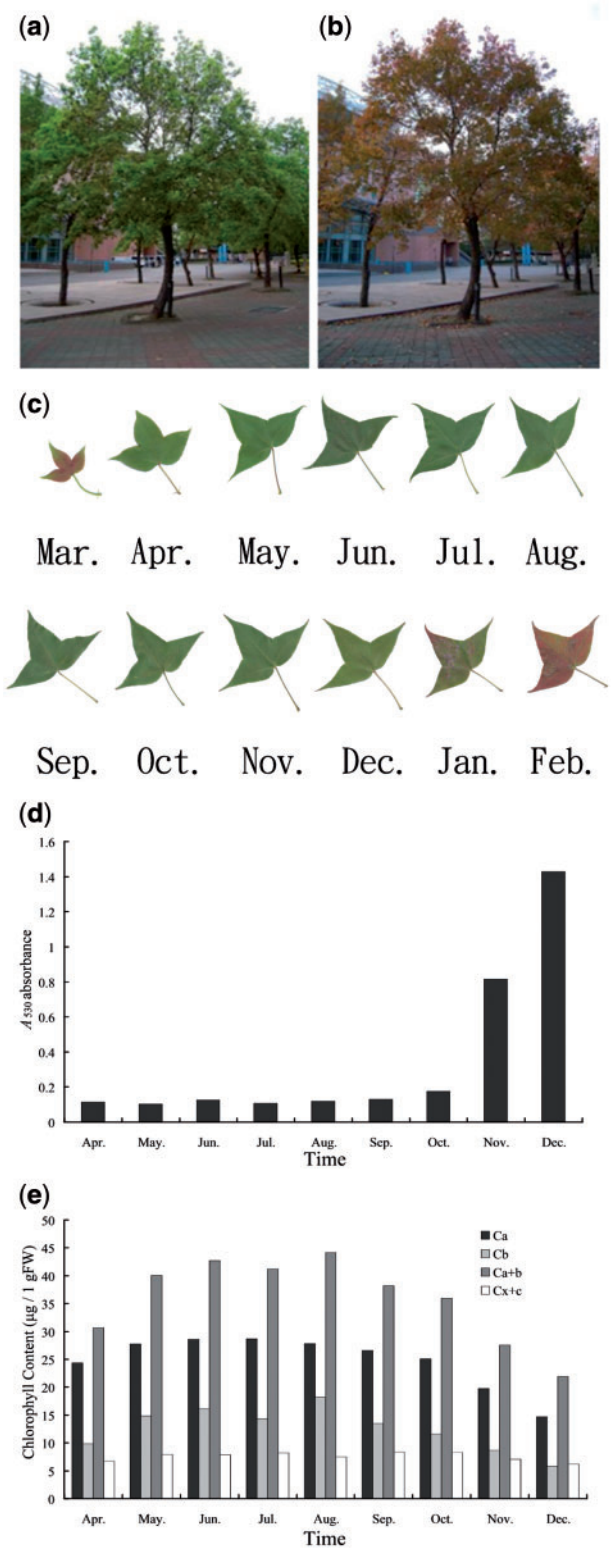

Fig. 1 Formosan gum leaf development and pigment quantification. (A) Scanned images of leaves from each month in 2011. (B) Sample tree in March 2013, showing developing leaves. (C) Sample tree in December 2013, showing leaves senescing. (D) Chl content averaged for 2011–2013. At least two repeats were performed each year. (E) Anthocyanin content averaged for 2011–2013. At least two repeats were performed each year.

their reference contigs (**Supplementary Fig. S1B**). When the amino acid sequences of the complete contigs were used for calculations, almost 4,000 contigs showed nucleotide identities of around 35–55% (**Supplementary Fig. S1C**). The ORFs of complete contigs have a length distribution similar to that of their reference contigs (**Supplementary Fig. S1D**), and the correlation coefficient was 0.986 (**Supplementary Fig. S1E**). Based on these findings, the complete contigs highly resembled their reference contigs, thus making them reliable.

Gene ontology annotation and analysis

To provide gene function classification, GO analysis was performed by Blast2GO software (Conesa et al. 2005). Of the 58,402 contigs, 8,687 (14.9%) were annotated to all three domains, and 38,149 (65.3%) were unannotated (**Supplementary Fig. S2**). For most of the groups, more contigs were annotated from the 'R' group than from the 'G' group (**Supplementary Fig. S3A**). This may be because the reads from 'R' contributed 51.4% of the reads using de novo assembly. More contigs were assigned the gene ontology (GO) terms 'cell growth' and 'plastid' in 'G' than in 'R'. When 'G' was used as the control, most of the contigs annotated were up-regulated (**Supplementary Fig. S3B**). However, among contigs that were assigned the GO terms 'cell growth' and 'plastid', more contigs were down-regulated than up-regulated. For other GO terms such as 'anatomical structure morphogenesis', 'lipid metabolism' and 'thylakoid', more contigs were down-regulated than up-regulated. In some GO term categories, most of the contigs annotated were up-regulated. Such categories included 'metabolism', 'translation', 'binding', 'catalytic activity', 'RNA binding', 'structural molecular activity', 'translation factor activity', 'cytoplasm' and 'ribosome'. Overall, the GO analysis indicated that there were many biological processes and molecular functions active in red leaf. This may indicate active metabolism of senescent leaves.

Chl and anthocyanin metabolism

Chl degradation and anthocyanin biosynthesis are the major contributors to the two major color-related events in autumn leaf senescence (Archetti et al. 2009). Therefore, the expression of genes participating in these two pathways was profiled (**Figs. 3**, **4**). The microarray showed that the expression patterns of some genes in Chl biosynthesis including LfGR21937 (*LfHEMD*), LfGR14678 (*LfHEME1*), LfGR1030 (*LfHEMF1*), LfGR7359 (*LfChlM*) and LfGR8427 (*LfPORA*) are correlated to Chl content. Some other genes involved in the steps of Chl biosynthesis showed a decline in expression after April, but had elevated expression again in October. These genes included LfGR1321 (*LfHEMB1*), LfGR9106 (*LfHEMBS*), LfGR73 (*LfCRD1*), LfGR11180 (*LfPORC1*), LfGR9423 (*LfDVR*) and LfGR2478 (*LfChlG*). Still other genes, including LfGR4958 (*LfGSA2*), LfGR206 (*LfChlI*) and LfGR1448 (*LfChlD*), were expressed at high levels in April, and then declined through the rest of the year. Most genes participating in the Chl *a*/*b* cycle and Chl degradation showed expression patterns that were opposite to those of the genes in the biosynthesis pathway. LfGR3207

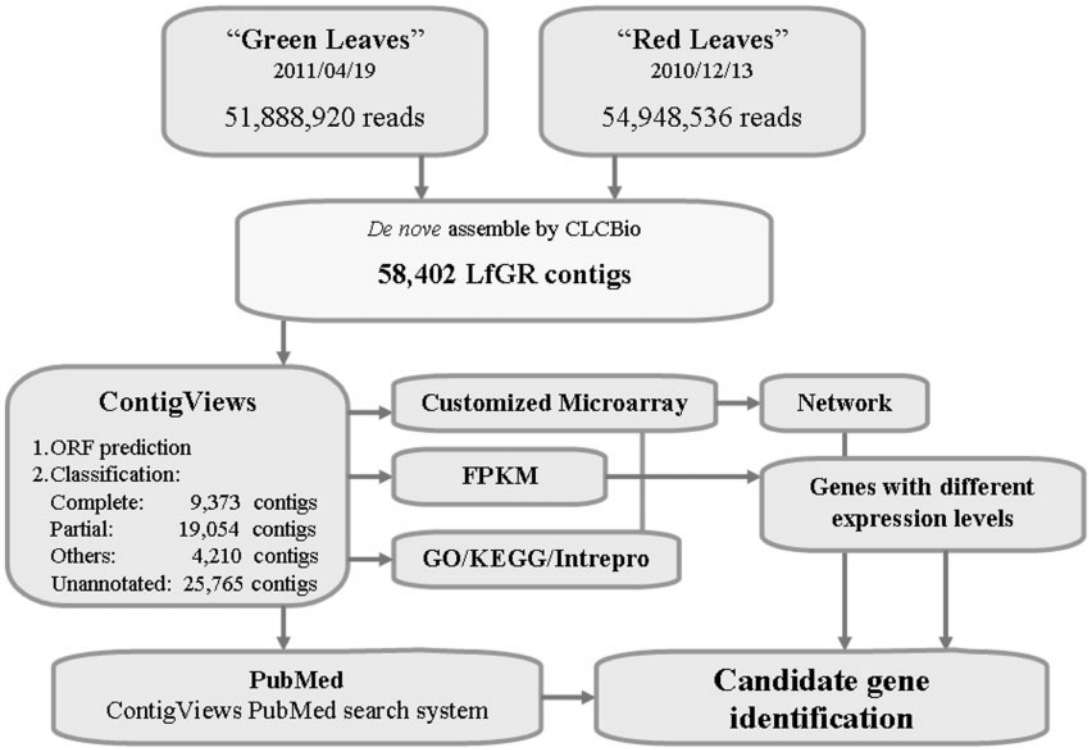

Fig. 2 Formosan gum leaf whole-transcriptome profiling workflow. Samples of green leaves (G) and red leaves (R) were collected individually from *Liquidambar formosana*. The contigs were classified based on the integrity status of the predicted open reading frame (ORF). FPKM, fragments per kilobase of transcript per million mapped reads; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; Interpro, Interpro domain database.

(*LfCAO1*), LfGR527 (*LfCAO2*) and LfGR6045 (*LfHCAR*) were all expressed at high levels in December. Among the Chl degradation-associated genes, some genes, particularly LfGR3426 (*LfPaO1*), LfGR17542 (*LfPaO2*) and LfGR10032 (*LfPaO3*), showed gradual up-regulation as autumn leaf senescence proceeded; however, LfGR2009 (*LfACD1-like1*) and LfGR1872 (*LfACD1-like2*) showed an opposite pattern.

In the anthocyanin biosynthesis pathway, most of the genes were expressed at low levels from June to August and expressed at high levels in December (**Fig. 4**). Some of the above, including LfGR4825 (*LfPAL1.1*), LfGR2255 (*LfPAL1.2*), LfGR3450 (*LfPAL2*), LfGR1674 (*LfC4H1*), LfGR2534 (*LfC4H2*), LfGR1093 (*Lf4CL1.1*), LfGR3811 (*Lf4CL1.2*), LfGR275 (*LfCHS1*), LfGR2663 (*LfF3′H1*), LfGR (*Lf3′H2*) and LfGR290 (*LfF3H*), also showed high expression in April. The four *LfDFR* homologs showed different expression patterns, and LfGR702 (*LfLDOX*) was expressed at a higher level in April than in December. The five *LfUF3GT* genes (LfGR6008, LfGR236, LfGR5914, LfGR3025 and LfGR7627) also showed different expression patterns from each other. Taken together, the expression of these genes correlated with anthocyanin content, being expressed at high levels when anthocyanin accumulated. This pattern was particularly pronounced for the genes encoding the limiting enzymes in the pathway; the *LfPAL* and *LfDFR* homologs. However, the relative expression levels of these genes did not exactly match the relative anthocyanin content, as LfGR4825 (*LfPAL1.1*), LfGR1093 (*Lf4CL1.1*), LfGR520 (*LfCHS3*) and LfGR352 (*LfDFR1*) were expressed at higher levels in April than in December, despite the low anthocyanin content.

Leaf senescence gene network

To search for the regulatory genes that participate in Formosan gum leaf senescence, all the contigs were cluster analyzed using the *k*-means algorithm. Contigs in cluster 4 which showed up-regulation after October were chosen for further analysis (**Supplementary Fig. S4**). In addition, the contigs with expression levels showing a 2-fold change between April and December were selected, and 160 of the above which were annotated with GO:0003700 (sequence-specific DNA-binding transcription factor activity) were used. After that, a network representing genes up-regulated during Formosan gum leaf senescence including 1,401 contigs was formed (**Fig. 5**). For searching the putative key regulators, betweenness centrality was used to calculate the value which represent how often a node appears in the shortest path between any other two nodes. Most of the contigs which had high betweenness centrality were annotated with GO:0003700, so they were putative transcription factors. These contigs included LfGR16612 (*LfWRKY70*), LfGR38306 (*LfWRKY65*), LfGR38864 (*LfNAC100*), LfGR40447 (putative heat shock factor B2A), LfGR49980 (putative NB-ARC), LfGR20995 (putative heat shock factor B3), LfGR21757 (*LfNAC1*), LfGR49959 (*LfMYB113*), LfGR3349 (*LfSPL14*) and LfGR16163 (putative nuclear factor Y subunit A2). We presume that these genes have major regulatory roles in Formosan gum leaf senescence. However, an exception was LfGR53927, which had high betweenness centrality but was annotated as a hypothetical protein and connected with many

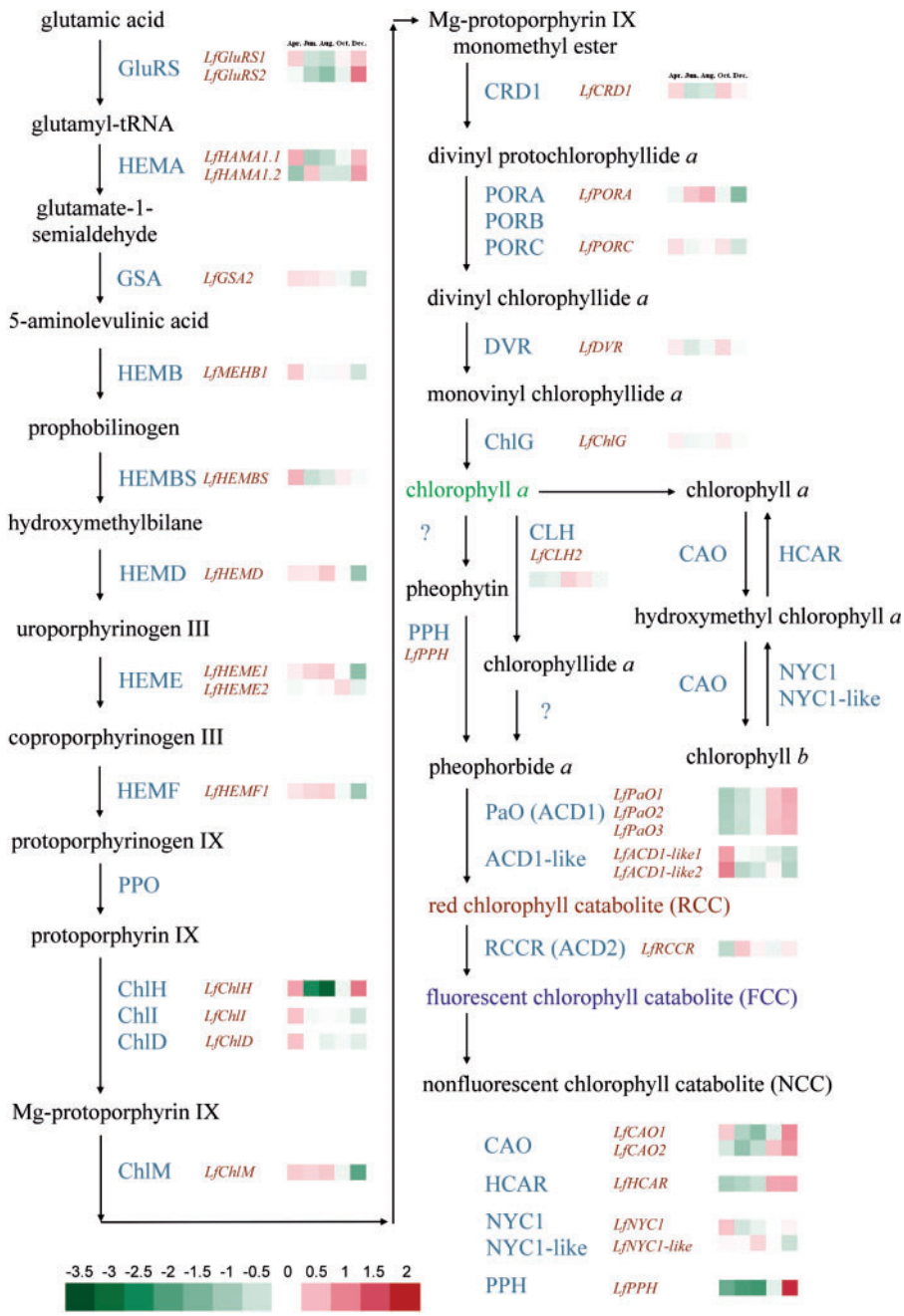

Fig. 3 Microarray gene expression of Formosan gum Chl metabolism enzymes. The five color box represents gene expression in April, June, August, October and December. Log_2 fold change was correlated to the median of the 5 months. The Chl metabolism enzymes were referenced to the pathway in Arabidopsis (Tanaka and Tanaka 2006, Schelbert et al. 2009).

short partial contigs in the network. One of the four contigs which was annotated as a senescence-associated gene (SAG), LfGR34509 (*LfSAG24*), was connected with LfGR53927. The other three senescence-associated genes, LfGR1304 (*LfSAG101.1*), LfGR8195 (*LfSAG101.2*) and LfGR26433 (*LfSAG1*), were connected with LfGR16612 (*LfWRKY70*), LfGR38306 (*LfWRKY65*), LfGR38864 (*LfNAC100*), LfGR40447 (putative heat shock factor B2A), LfGR16163 (putative nuclear factor Y subunit A2) and LfGR10544 (putative DNA-binding protein), suggesting that these putative transcription factors regulate leaf senescence genes.

Identification of candidate regulatory genes involved in autumn leaf coloration

To illustrate the connection between senescence and coloration, the contigs which had high betweenness centrality to the contigs coding Chl degradation or anthocyanin biosynthesis enzymes were searched. A subnetwork was extracted from the 1,401 contigs (**Fig. 6**). LfGR9306 (*LfPPH*) was the only Chl degradation enzyme connected to the regulatory genes in this network; it was connected to LfGR49959, LfGR20995, LfGR21757, LfGR20567 and LfGR22722. Two *SGR* homologs were found,

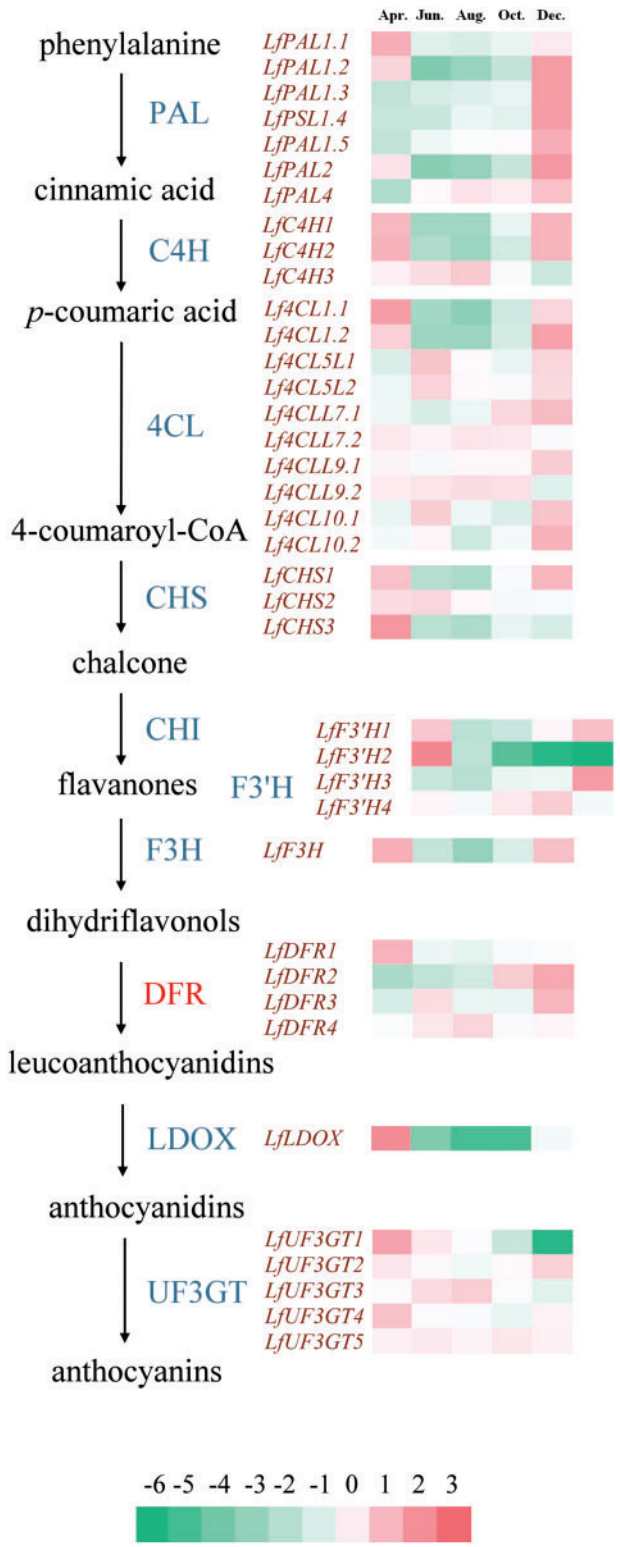

Fig. 4 Microarray gene expression of Formosan gum anthocyanin biosynthesis enzymes. The five color box represents gene expression in April, June, August, October and December. Log_2 fold change was correlated to the median of the 5 months. The anthocyanin biosynthesis pathway was simplified from the pathway in Arabidopsis (Winkel-Shirley 2001).

LfGR19314 and LfGR51156, which were homologs of *SGR1* and *SGR2*, respectively. LfGR19314 (*LfSGR1*) and LfGR51156 (*LfSGR2*) were connected to four major regulators, LfGR16612, LfGR21757, LfGR20995 and LfGR49959. LfGR16612, LfGR21757, LfGR38864, LfGR38306 and LfGR17486 form a subnetwork composed of the *WRKY* and *NAC* families. This subnetwork connected to LfGR40622 (*LfDFR2*), which was annotated as one of the anthocyanin biosynthesis pathway genes but was classified as 'others' due to the stop codons in the ORF. Further sequence confirmation of *LfDFR2* was conducted using rapid amplification of cDNA ends (RACE) and reverse transcription–PCR (RT–PCR), proving it had a complete ORF (**Supplementary Fig. S5**). The other four regulators participating closely with the *WRKY*, *NAC* and *LfSGR* genes were LfGR49980, LfGR20995, LfGR40447 and LfGR20567 (a putative integrase-type DNA-binding superfamily protein). Although currently the function and regulatory roles of these genes are not clear, one of the four, LfGR40447, showed a connection with LfGR43065 (*LfPAL1.5*). Based on the connection between these contigs, we suggest that the network illustrated in **Fig. 6** is a central regulatory network that connects leaf senescence and coloration.

Discussion

This work profiles the gene expression of leaf development of the Formosan gum over a whole year with three biological repeats (2011–2013). Contig annotation, including ORF prediction, function prediction and GO terms, was acquired through bioinformatics analysis using ContigViews. The 3 year time series gene expression profile was monitored by microarray, which was designed based on sequences from the NGS. After gene expression network analysis, similar key regulators were compared across species from annuals to perennials. A new regulation network was identified that indicates the link between autumn leaf senescence and coloration. To our knowledge, this is the first time autumn leaf senescence has been studied from leaf development to coloration in three biological replicates in a subtropical deciduous plant, and also the first time autumn leaf coloration has been connected to a central leaf senescence gene regulation network.

Subtropical deciduous autumn leaf senescence

In this work, a succinct Formosan gum leaf development time line was outlined. To date, the strictest cellular timetable of autumn senescence recorded was for aspen (*Populus tremula*) (Keskitalo et al. 2005). Based on the definitions from Keskitalo et al. (2005), leaf senescence in Formosan gum starts in September, as marked by a decline in Chl content (**Fig. 1E**). Although it is a subtropical plant, the onset of Formosan gum autumn leaf senescence in this study is probably around the same time as in the temperate aspen. The coloration caused by anthocyanin acculmulation started around October and November (**Fig. 1D**), about 2 months later than onset in aspen in September (Keskitalo et al. 2005). The duration of Formosan gum autumn leaf senescence from September to

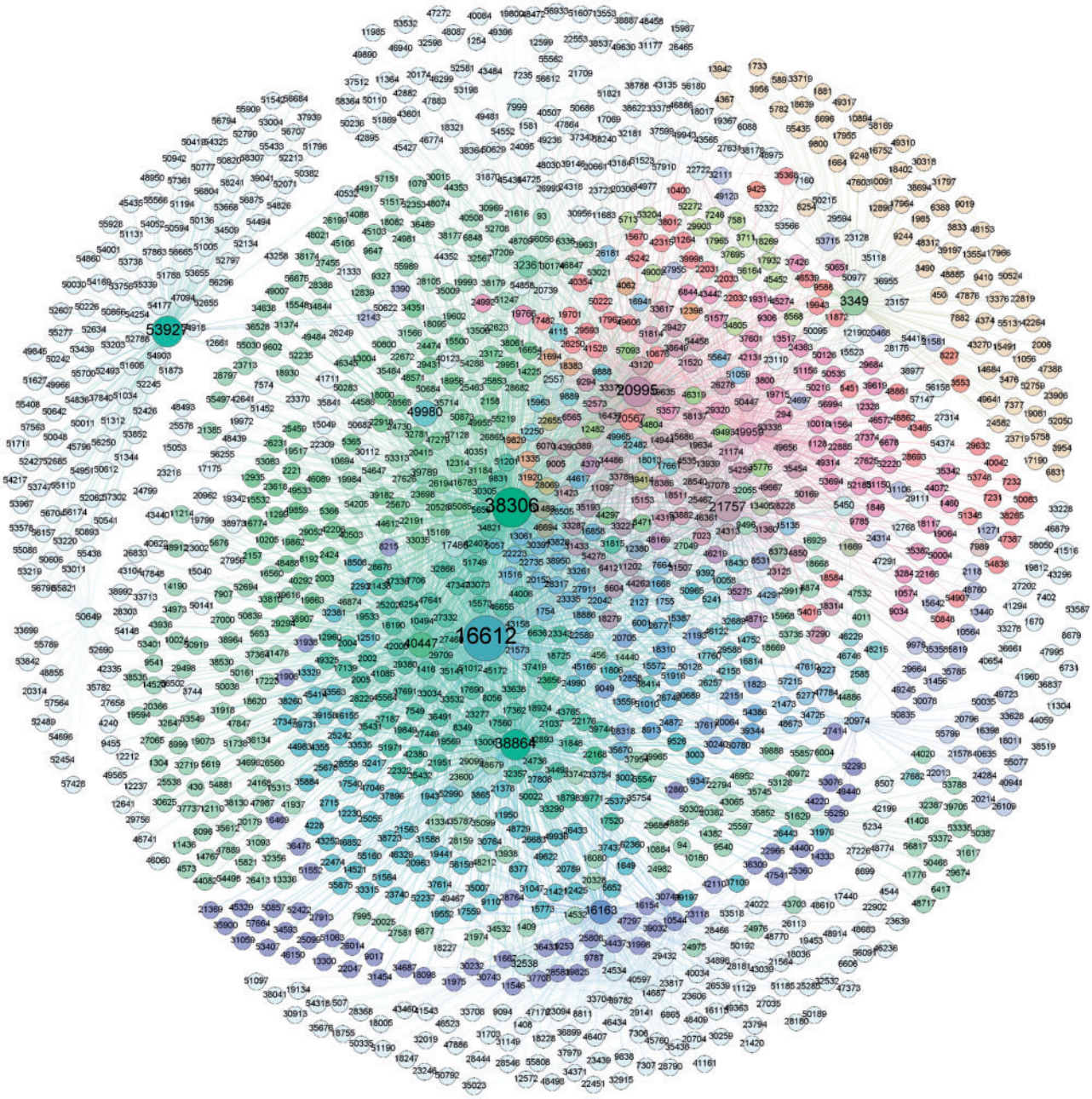

Fig. 5 Network of genes up-regulated in autumn leaf senescence of Formosan gum. Every circle represents a contig; numbers represent contig serial numbers in the database. The size and green color of the circle represent the degree of betweenness centrality. The blue circles mark contigs connected to LfGR38864 (*LfNAC100*), the red circles mark contigs connected to LfGR49959 (*LfMYB113*) and the orange circles mark contigs connected to LfGR3349 (*LfSPL14*). The colors of the circles are merged. The image was produced using Gephi (Bastian and Heymann 2009).

February the following year is longer than that of autumn leaf senescence in aspen, but the major molecular events progress in the same sequence in both plants.

The lifetime of Formosan gum leaves was about 11 months, in comparison with the lifetime of the fourth rosette leaves of the herbaceous plant Arabidopsis of about 34 d (Woo et al. 2013). The Chl degradation pattern of Formosan gum was remarkably similar to that of Arabidopsis, with an elongated time scale (Breeze et al. 2011). Since the major regulators identified in Formosan gum, for example *LfWRKY70*, were homologs to key leaf senescence regulators in Arabidopsis, we speculate that the autumn leaf senescence regulatory network is highly conserved across species. Both of the *LfNAC* genes (LfGR21757 and LfGR38864) which had the best BLAST score with *NAC1* (AT1G56010.2) and *NAC100* (AT5G61430) in TAIR had a putative *miR164* binding site. *miR164* is a microRNA that regulates leaf senescence through suppressing *ORESARA1* (*ORE1*, *ANAC092*, AT5G39610) as the leaf develops and declines when leaves undergo senescence (Kim et al. 2009, Woo et al. 2013). *miR164* is highly conserved across species from monocots

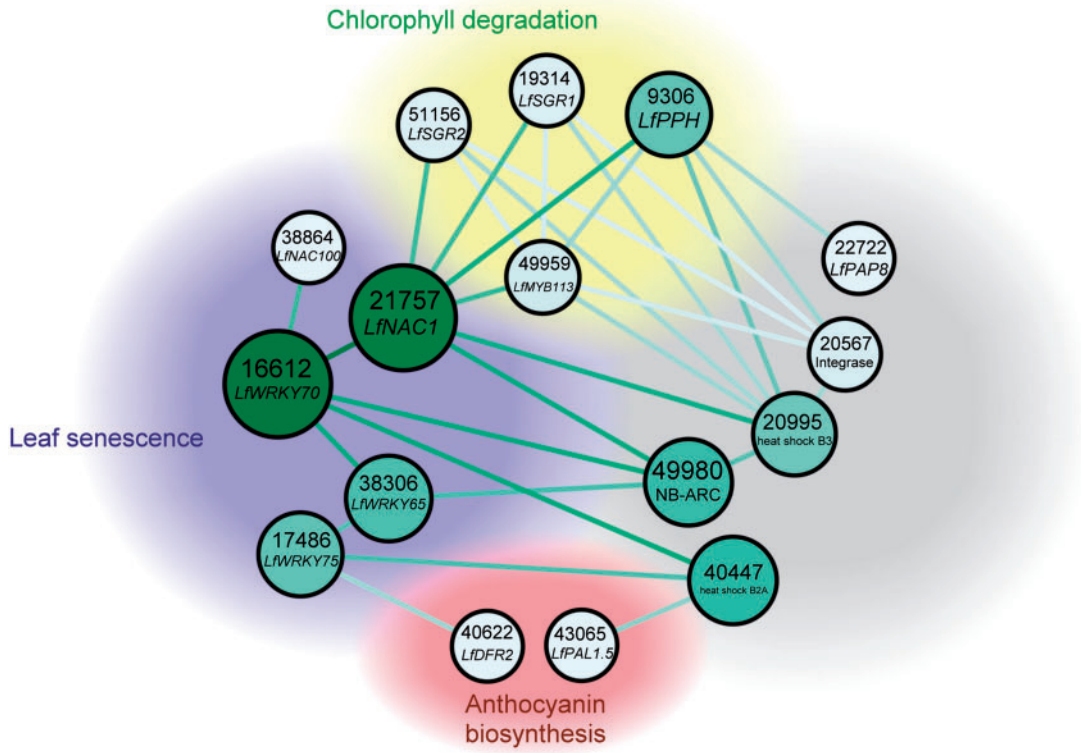

Fig. 6 Simplified putative gene network showing the link between autumn leaf coloration and senescence. Numbers in circles are the contig serial numbers. Below the numbers are gene names or putative functions. The network image was produced using Gephi (Bastian and Heymann 2009).

to dicots and perennials (Jones-Rhoades et al. 2006). Formosan gum LfGR21757 (*LfNAC1*) and LfGR38864 (*LfNAC100*) may have regulatory roles in leaf senescence under the regulation of *miR164*, in the same manner as *ORE1* in Arabidopsis.

Chl and anthocyanin metabolism regulation

Chl degradation and anthocyanin biosynthesis contribute to the development of autumn colors, and mark the onset and progression of autumn leaf senescence (Keskitalo et al. 2005, Ougham et al. 2005). All the gene members homologous to those in the Chl metabolic pathway were identified in this work except for protoporphyrinogen IX oxidase (PPO) (**Fig. 3**). Most of the biosynthesis genes were up-regulated before August and down-regulated thereafter. Most of the degradation genes showed opposite expression patterns to the biosynthetic genes, correlating with the Chl content and suggesting that Chl degradation is regulated at the transcriptional level in Formosan gum leaf senescence.

The key enzyme for loss of green color in leaves during senescence is PaO, which opens the porphyrin macrocycle of pheophorbide *a* by coupling with red chlorophyll catabolite reductase (RCCR) and also acts as a control point in the overall regulation of Chl degradation (Pruzinská et al. 2003, Chung et al. 2006, Aubry et al. 2008). Since *PaO* was identified, many studies have focused on its regulation, but have failed to provide molecular verification of the direct regulators. In our gene expression network analysis, the three *LfPaO* genes formed a subgroup and did not show connections with other genes. The regulation of *PaO* may be at the post-translational level (Pruzinská et al. 2003). Currently PaO is indicated to be in a complex with SGR and five chlorophyll catabolic enzymes (CCEs) (Sakuraba et al. 2012). The CCEs are NONYELLOW COLORING1 (NYC1), NYC1-LIKE, pheophytin pheophorbide hydrolase (pheophytinase, PPH), RCCR and light-harvesting complex II (LHCII) (Sakuraba et al. 2012). Although the exact role of *SGR* is still unclear, it is required for initiation of Chl breakdown and is essential for recruiting CCEs in senescing chloroplasts (Sakuraba et al. 2012).

In addition to PaO, PPH has been reported to be another key enzyme involved in Chl degradation. PPH is speculated to be the major enzyme responsible for dephytylation, a reaction that was previously thought to be catalyzed by chlorophyllase (CAO) (Schelbert et al. 2009, Büchert et al. 2011, Hörtensteiner 2013). In addition, the *pph* mutant of Arabidopsis results in a stay-green phenotype (Schelbert et al. 2009). In Formosan gum, *LfPPH* was up-regulated when leaves underwent senescence and showed a connection with five genes in the network analysis. Two of the genes connected to *LfPPH* were annotated to be key transcription factors in leaf senescence, including *LfNAC1* and *LfMYB113*, indicating that they are putative regulators of Chl degradation in autumn leaf senescence (**Figs. 5**, **6**). The remaining gene including *LfPAP8* (Lf22722), which was annotated as purple acid phosphatase. Purple acid phosphatase

catalyzes the hydrolysis of a wide range of phosphate esters and was suggested to control the activity of α-xylosidase and β-glucosidase (Kaida et al. 2010). However, its function in leaf senescence is unclear.

Anthocyanins are flavonoids that are responsible for the color of vegetative organs (Winkel-Shirley 2001). The major contributor of red color in autumnal senescing leaves is the anthocyanin cyanidin-3-glycoside (Ishikura 1972, Lee et al. 2003). In Formosan gum, cyanidin-3-glycoside content correlated to the red pigment in the leaves in the scanned images (**Figs. 1C**, D). Of the enzymes in the anthocyanin biosynthesis pathway, the only one not found in our analysis was chalcone isomerase (CHI). The expression levels of genes in the anthocyanin biosynthesis pathway were up-regulated during leaf senescence, but some of them also showed high levels of expression in April (**Fig. 4**). This may be due to the production of different anthocyanin products in April and December. Different anthocyanin products were found in developing and senescing leaves in *Acer polmatum* and *Rhus succedanea* (Ishikura 1972).

The regulation of anthocyanin biosynthesis has been studied across many species from annuals to perennials, and the MBW complex has been shown to be a highly conserved regulatory mechanism (Lin-Wang et al. 2010, Jaakola 2013, Albert et al. 2014). Among the major regulatory genes identified in the network analysis, LfGR49959 (*LfMYB113*) showed 81.65% identity to *AtMYB113* (AT1G66370) and was connected to major regulators LfGR21757 (*LfNAC1*) and LfGR20995 (putative heat shock factor B3). In cluster 4, LfGR34491 and LfGR31588 were bHLH (basic helix–loop–helix) transcription factors, showing the best BLAST hits in TAIR to AT2G22770 and AT4G37850, respectively. They connected to LfGR16163 (putative nuclear factor Y, subunit A2), LfGR16612 (*LfWRKY70*), LfGR38864 (*LfNAC100*) and LfGR40447 (heat shock factor B2A). From the WD repeat gene family, *TTG1* (AT5G24520) in Arabidopsis has been illustrated to have a regulatory role in anthocyanin biosynthesis (Gonzalez et al. 2008). In Formosan gum, LfGR8160 is the putative homolog of *TTG1*, but it was not included in cluster 4. The other two members of the WD repeat gene family found in the network were LfGR10894 and LfGR16752. The best BLAST hits in TAIR showed LfGR10894 to have 58.52% identity to AT5G24320.2, and LfGR16752 to have 75.85% identity to AT1G24530.2. Both were connected to LfGR3349 (*LfSPL14*) in the network. *LfMYB113* was the most conserved anthocyanin biosynthesis regulator. However, *LfMYB113* did not show a direct connection with the anthocyanin biosynthesis genes. As its orthologs in other species have been demonstrated to be up-regulated during leaf senescence (Lin-Wang et al. 2010, Butelli et al. 2012), we speculate that *LfMYB113* may play an important role in anthocyanin regulation in Formosan gum autumn leaf senescence.

Putative network between autumn leaf coloration and senescence

The regulation of autumn leaf coloration and senescence has long been an enigma. Many transcription factors have been identified to play important roles in leaf senescence, including members of the *NAC* and *WRKY* gene families (Lim et al. 2003). In Formosan gum autumn leaf senescence gene expression network analysis (**Fig. 6**), contigs annotated to these two families were identified as major regulators. In the *NAC* gene family, there were LfGR21757 (*LfNAC1*) and LfGR38864 (*LfNAC100*). LfGR16612 (*LfWRKY70*) was identified as a homolog of *AtWRKY70* (AT3G56400.1). *AtWRKY70* is a negative regulator of leaf senescence, which gradually increases in expression and reaches a maximum at leaf senescence onset (Besseau et al. 2012). *LfWRKY70* expression showed the same pattern, but its expression level continued to increase as the leaf underwent senescence. The other two *WRKY* members, LfGR38306 (*LfWRKY65*) and LfGR17486 (*LfWRKY75*), were annotated to *AtWRKY65* (AT1G29280.1) and *AtWRKY75* (AT5G13080.1). *AtWRKY65* was up-regulated in Arabidopsis suspension culture cells in response to sucrose starvation, and *AtWRKY75* was induced during phosphate deprivation (Contento et al. 2004, Devaiah et al. 2007). Up-regulation of *LfWRKY65* and *LfWRKY75* may indicate that the leaf was under nutrient stress as autumn senescence progressed. As in Arabidopsis, many stress-related regulators were up-regulated in leaf senescence (Nam 1997, Lim et al. 2003).

Four genes showed a connection to *LfMYB113*, *LfWRKY70*, *LfWRKY75*, *LfWRKY65* and *LfNAC1*: LfGR40447 (annotated to AT5G62020.1, putative heat shock factor B2A), LfGR49980 (AT1G50180.1, putative NB-ARC domain-containing disease resistance protein), LfGR20995 (AT2G41690.1, putative heat shock factor B3) and LfGR20567 (AT5G07310.1, *Ethylene Response Factor 115*, *ERF115*, putative integrase-type DNA-binding superfamily protein). LfGR40447 and LfGR49980 represent stress response-related regulation in autumn leaf senescence, as heat shock proteins protect plant cells against many stresses (Timperio et al. 2008). LfGR49980 also probably plays a stress resistance role. According to domain prediction by ContigViews, LfGR20567 contains an AP2-ERF domain. Since it has been suggested that *ERF115* in Arabidopsis has a role in the cell cycle regulatory mechanism (Heyman et al. 2013), LfGR20567 may be an important regulator in Formosan gum leaf senescence.

The key enzymes that provide substrates for downstream metabolic pathways in autumn leaf coloration were included in the network. LfGR9306 (*LfPPH*) is an enzyme that provides pheophorbide for PaO. LfGR43065 (*LfPAL1.5*) and LfGR40622 (*LfDFR2*) are rate-limiting enzymes in anthocyanin biosynthesis. In addition, LfGR19314 (*LfSGR1*) and LfGR51156 (*LfSGR2*) may have roles in the regulation of Chl degradation. There was no direct connection between the Chl degradation genes and anthocyanin biosynthesis genes, but both showed connections to the highly conserved leaf senescence regulators.

In conclusion, the putative regulatory network presented in this study provides a link between the central regulators in leaf senescence and the key genes participating in leaf coloration. In spite of the fact that only gene expression correlations are represented, the network analysis implies that these genes have direct or indirect relationships with each other. This

work constructed a subtropical deciduous, Formosan gum leaf transcriptome database on a 3 year time series scale and described the putative genes participating in autumn leaf coloration.

Materials and Methods

Leaf material

Leaves from Formosan gum (*Liquidambar formosana* Hance) were sampled in the third or fourth week of each month from December 2010 to December 2013 on the campus of Taiwan National University, Taipei, Taiwan. A free-growing tree was chosen and 20 leaves from two or three south-facing branches were collected. Two of the leaves were used for scanning; the others had the petiole detached and were pooled, frozen and fragmented using liquid nitrogen. All the samples were stored at −80°C before use.

Transcriptome sequencing

Samples collected on December 13, 2010 and April 19, 2011 were named 'R' and 'G', respectively. Total RNA was extracted using Concert Plant RNA Reagent (PureLink, Invitrogen Life Technologies), followed by DNase I (Ambion, Life Technologies) treatment, purified by ethanol precipitation and quality controlled by Agilent 2100 Bioanalyzer (Agilent Technologies). Library construction and sequencing was performed using Illumina HiSeq 2000 (Genomics BioSci & Tech Co.). The paired-end reads from 'R' and 'G' were assembled and pooled together using the de novo assembly program in CLC Genomics Workbench 5 (CLC bio). All the reads and contigs were analyzed and a database was constructed in ContigViews web server (http://contigviews.csbb.ntu.edu.tw). The ORF and GO annotation were determined using the rule-based predictor and Blast2GO built into ContigViews (Liu et al. 2014). The identity was calculated using EMBOSS Needle (McWilliam et al. 2013).

Microarray experiment

All 58,402 contigs were used to design customized microarray probes, and 58,355 successfully passed the probe design requirement and were synthesized as 60-mer oligonucleotides (Agilent Technologies). Samples collected in December 2010, as well as in those collected every April, June, August, October and December from 2011 to 2013 were prepared for microarray. A time series through each year was defined as a biological repeat. Total RNA was extracted using the Pine Tree method (Chang et al. 1993). The microarray experiments were performed by the Institute of Plant and Microbial Biology DNA Microarray Core Laboratory (Academia Sinica). In brief, 0.2 μg of total RNA was amplified by a Low Input Quick Amp Labeling kit (Agilent Technologies) and labeled with Cy3 (CyDye, Agilent Technologies) during the in vitro transcription process. Cy3-labeled cRNA (0.6 μg) was fragmented at 60°C for 30 min. Correspondingly fragmented labeled cRNA was then pooled and hybridized to the Agilent Custom design 8×60K Microarray (Agilent Technologies) at 65°C for 17 h. After the washing and drying steps, the microarrays were scanned with an Agilent microarray scanner (Agilent Technologies) at 535 nm for Cy3. The array image was analyzed by the Feature Extraction software version 10.7.1.1 using the default setting.

Network analysis

The gene expression data used for network analysis were generated from microarray data using GeneSpring software version 11.5. After normalization, averaged by month, all the features were filtered by flag as the features in any month used for further analysis had to be detected in all three years. A *k*-means algorithm was used to cluster the features into five groups (**Supplementary Fig. S4**). There were 6,104, 1,339, 21,942, 8,164 and 2,816 contigs listed from cluster 0 to cluster 4, respectively. To list the query contigs annotated as putative transcription factors, all the features after filtering were used and those which showed a 2-fold change between April and December were extracted (April and December intensity ratio ≥ 2 or ≤ 0.5), then all those which were annotated to GO:0003700 were extracted. A total of 160 contigs were extracted and listed as query entities.

The network analysis was conducted using algorithms based on the R program built into ContigViews (Liu et al. 2014). First, analysis of variance (ANOVA) was used to define differentially expressed genes. Each *k*-mean cluster was used as a query entity to calculate the correlation network, and each contig was considered as a node. If a correlation coefficient of ≥ 0.9 (or ≤ -0.9) was obtained, the two nodes were connected. The network images (**Supplementary Fig. S6**) were produced using Gephi (Bastian and Heymann 2009).

Pigment quantification

Chl quantification was according to the methods described for Arabidopsis (Breeze et al. 2011) and *Quintina serrata* A.Cunn. (Gould et al. 2000). Anthocyanin absorbance measurement was according to methods for sugar maple (*Acer saccharum* Marsh.) (Schaberg et al. 2008). From April, 2011 to December, 2013, samples were collected each month in the growing seasons, two repeats were performed from the same month and values from the 3 years were averaged. Extracted materials (200 μl) were transferred to a quartz 96-well plate (Hellma) and the absorbance was measured on a μQuant Universal Microplate Spectrophotometer (BioTek).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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