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Dissecting the 'bacon and eggs' phenotype: transcriptomics of post-anthesis colour change in *Lotus*

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• **Background and Aims** Post-anthesis colour change (PACC) is widely thought to be an adaptation to signal floral suitability to pollinators. *Lotus filicaulis* and *Lotus sessilifolius* are insect-pollinated herbaceous legumes with flowers that open yellow, shift to orange and finally red. This study examines the molecular basis for floral colour change in these *Lotus* species.
• **Methods** *Lotus filicaulis* was cultivated in a glasshouse from which pollinating insects (bees) were excluded, and the rate of colour change was recorded in both unpollinated and manually pollinated flowers. Unpollinated flowers from both the yellow stage and the red stage were sampled for sequencing. The transcriptomes of *L. filicaulis* and *L. sessilifolius* of both colour stages were analysed for differentially expressed genes and enriched ontologies.
• **Key Results** The rate of progression through PACC doubled when *L. filicaulis* was hand-pollinated. *De novo* assembly of RNA-Seq reads from non-model *Lotus* species outperformed heterologous alignment of reads to the *L. japonicus* genome. Differential expression analysis suggested that the carotenoid biosynthetic pathway is upregulated at anthesis while the flavonoid biosynthetic pathway is upregulated with the onset of PACC in *L. filicaulis* and *L. sessilifolius*.
• **Conclusion** Pollination significantly accelerates PACC in *L. filicaulis*, consistent with the hypothesis that PACC increases pollination efficiency by directing pollinators to unpollinated flowers. RNA-Seq results show the synchronized upregulation of the entire cyanidin biosynthesis pathway in the red stage of PACC in *L. filicaulis* and *L. sessilifolius*. The genes implicated offer the basis for further investigations into how gene families, transcription factors and related pathways are likely to be involved in PACC.

Key words: Post-anthesis colour change, anthocyanins, transcriptomics, pollination, *Lotus*.

INTRODUCTION

In angiosperms, flowers are typically left on display until pollination or senescence (i.e. until the pollen becomes inviable or the stigma non-receptive), at which time the flower will generally wilt and cease reward production. Often the corolla abscises at this point, thus effectively excluding any further visits from pollinators (Faegri and Vanderpijl, 1979). However, instead of abscission, some species retain the corolla past the cessation of reward production and modify petal colour, a trait we herein refer to as post-anthesis colour change (PACC) (Mohan Ram and Mathur, 1984; Farzad *et al*., 2003). We use 'post-anthesis colour change' instead of the broader 'floral colour change' as many flowers change colour between pre-anthesis (bud) and anthesis (flower opening). This early-maturation colour change is unrelated to PACC. Several species of *Lotus* (Fabaceae) exhibit yellow flowers at anthesis followed by a transition to red (Ojeda *et al*., 2013); thus, these species have sometimes acquired the English vernacular name 'bacon and eggs' (Grigson, 1975) (Fig. 1). A well-studied example is the North American lotoid legume *Acmispon glaber* (formerly known as *Lotus scoparius*) (Jones and Cruzan, 1999).

The widespread (Weiss, 1995) and striking phenomenon of PACC has long interested botanists. One of the first scientific observations of PACC dates to a letter written from Fritz Müller to Charles Darwin, discussing the pollination ecology of *Lantana camara* (Müller, 1877; see also Weiss, 1991; Jones and Cruzan 1999). Müller notes that *L. camara* flowers open as yellow and over a 3-d period shift to orange and finally purple. Tellingly, he also writes that butterflies tend to visit yellow flowers most often, occasionally visiting the orange flowers, and never the purple flowers. More recently the phenomenon has been documented in a variety of studies and reviews (Mohan Ram and Mathur, 1984; Weiss, 1991; Ojeda *et al*., 2013; Brito *et al*., 2015).

As anticipated by Müller, PACC is likely an adaptation to redirect pollinators at close range while maintaining long-distance appeal of the plant floral display (Weiss, 1991; Jones and Cruzan, 1999), hence the retention of the corolla instead of wilting or abscission. Indeed, in the North American *Acmispon glaber* PACC is accelerated by pollination and corresponds temporally with the termination of reward production (Jones and Cruzan, 1999). By differentiating between young and older flowers, it is thought that plants are able to signal to pollinators

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FIG. 1. *Lotus filicaulis*, exhibiting post-anthesis colour change from yellow to red, a trait commonly referred to as the 'bacon and eggs' phenotype. Photograph taken at the Horticulture Glasshouse, University of British Columbia.

which flowers possess a food reward (Weiss, 1991). This is of potential benefit for both organisms as the pollinator forages with greater efficiency, while the plant may receive a higher rate of successful pollination events. Pollinators could, of course, be directed to young flowers by the wilting or abscission of old flowers, but it is hypothesized that PACC provides a selective advantage by aiding long-distance attractiveness of the overall floral display.

The flower colours involved in effective signalling are not random; PACC tends to be a shift from shorter reflective wavelengths (e.g. yellow) to longer ones (e.g. red). Like many floral traits, it is plausible that PACC has evolved through interaction with pollinators, and thus the characteristics of insect vision may be implicated in the colour palette of PACC. Generally, bees (Hymenoptera: Apidae) have three kinds of light receptors, with peak sensitivities at 340 nm (ultraviolet, S-receptor), 430 nm (blue, M-receptor) and 540 nm (green, L-receptor) (Chittka, 1996). However, the physiology of hymenopteran light receptors (Chittka, 1996) and colour-reward experiments (Weiss, 1991; Chittka and Waser, 1997; Martínez-Harms *et al*., 2010) suggest that bees are not incapable of detecting red light (620–750 nm), and electroretinogram recordings of *Bombus dahlbomii* (Apidae) have demonstrated that red light can induce an L-receptor response (Martínez-Harms *et al*., 2010). Indeed, in the wild *B. dahlbomii* is known to visit a variety of plant species with flowers that look red to humans, and they can be trained to visit artificial red targets *ex situ* (Martínez-Harms *et al*., 2010). Results of colour-reward experiments suggest that bees are able to detect red flowers by achromatic contrast (Chittka and Waser, 1997; Martínez-Harms *et al*., 2010). That is, red flowers likely appear as a dark, hue-less patches against a background of green plant foliage, providing sufficient contrast to locate such flowers (Martínez-Harms *et al*., 2010).

Given that a pollinator spends a finite amount of time at any one plant, PACC may be an adaptation to deceptively attract insect pollinators at long range by retaining flowers, regardless of condition, while honestly directing pollinators at short range to the flowers most likely to benefit from pollen transfer (discussed in Brito *et al*., 2015). In numerous plant taxa the visual physiology of pollinators has mediated the evolution of flower colour (Rausher, 2008); in the case of PACC it is conceivable that natural selection would favour a post-anthesis colour that is relatively less attractive than at anthesis, but not so unattractive as to be indistinguishable from its surroundings.

The timing of PACC is potentially tied to a host of environmental and biochemical triggers that influence pigment pathways in conjunction with the cessation of food rewards. In *Lantana camara*, *Gossypium hirsutum* and *Viola cornuta*, PACC has been found to result from changes in flavonoid biosynthetic processes (Mohan Ram and Mathur, 1984; Farzad *et al*., 2003; Tan *et al*., 2013). These are highly conserved pigment pathways known to produce the red and purple coloration in plant tissues. However, the regulation of PACC at the molecular genetic level remains poorly known. Additionally, many

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flavonoid biosynthetic pathway genes are represented as gene families with multiple duplicated copies (Ober, 2005) and whether all or some of these copies are involved in PACC remains to be determined.

Lotus is a genus with a diverse range of floral traits to attract pollinators (Ojeda *et al*., 2013), including varying sugar compositions, corolla shapes, petal micromorphologies and petal pigmentations (Ojeda *et al*., 2012a, 2013). It is a promising genus for studying the evolution of pollination syndromes, and PACC is particularly interesting because it has been suggested that it is a preadaptation (developmental 'pre-pattern') for the evolution of bird pollination in *Lotus* section *Rhyncholotus* (Ojeda *et al*., 2013). *Lotus* includes the well-studied *L. corniculatus*, which is of considerable economic importance as a forage crop (Duke, 1981) and exhibits PACC. However, *L. corniculatus* is a polyploid and therefore a complex organism for studying gene expression.

The anthocyanin pathway in *Lotus japonicus* K. Larsen (*L. corniculatus* L. var. *japonicus* Regel) has been well studied, but it does not exhibit PACC. Chromatographic analysis of *L. japonicus* aerial tissue has revealed that cyanidin and peonidin (*O*-methylated form of cyanidin) make up the flavonoid profile of this species (Suzuki *et al*., 2008); both of these pigments are known to reflect red and purple light and it is possible that this branch of the anthocyanin pathway is involved in the *Lotus* species that exhibit PACC. For this reason we have chosen to leverage the genomic resources available for *L. japonicus* by studying PACC in the closely related *Lotus filicaulis*, a diploid perennial herbaceous legume (Ferreira and Pedrosa-Harand, 2014), notable for its post-anthesis transition from yellow to red flowers (Fig. 1). For comparison we also investigate another species, *Lotus sessilifolius*, which has been the subject of previous studies (Ojeda *et al*., 2013) and may be important in the evolution of the bird pollination syndrome in *Lotus* (Ojeda *et al*., 2012b).

In this study we first investigate whether PACC is triggered by pollination in *L. filicaulis*. We then compare RNA expression at anthesis (yellow) and during PACC (red) in *L. filicaulis* and *L. sessilifolius* to discover the pigment pathways involved, and propose several candidate genes and pathways that we believe play a key role in post-anthesis colour change in these *Lotus* species. This is the first transcriptome-wide analysis of PACC to date. PACC appears to have evolved independently in *L. filicaulis* and *L. sessilifolius* (Ojeda *et al*., 2013) (Fig. 2) as they belong to different clades of *Lotus*. *Lotus sessilifolius* is in the Canary Island clade of *Lotus* (Ojeda and Santos-Guerra, 2011; Ojeda *et al*., 2013). However, *L. sessilifolius* (insect-pollinated) is the sister taxon to the bird pollinated 'rhyncholotus group' in *Lotus* (Ojeda *et al*., 2013), and further studies on this species can provide additional information about the evolution of bird pollination in this group. We discuss the results in the context of observations regarding PACC in other plant species, the role of pollinators in driving the evolution of PACC, the role of gene duplication, and known molecular pathways of pigment biosynthesis.

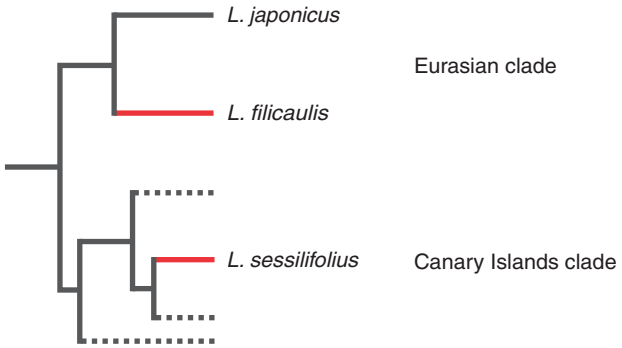

FIG. 2. Simplified phylogeny of the *Lotus* species used in this study (modified from Ojeda *et al*., 2013). Red branches show clades where PACC has evolved and dotted branches indicate the presence of adjacent clades.

MATERIALS AND METHODS

Plant material and cultivation

The North African species *Lotus filicaulis* (voucher: *Ojeda 71*, herb. UBC) was used for experiments on floral manipulation; *L. filicaulis* is sometimes regarded as synonymous with *L. tenuis*. A second species, *Lotus sessilifolius* (voucher: *Ojeda 225*, herb. UBC) from the Canary Islands, Spain, was added as a comparison for RNA-Seq experiments. Both species exhibit strong PACC with a yellow to red colour change. The plants were propagated from seeds in the horticulture glasshouse, University of British Columbia, Vancouver, Canada. All plants were grown in pots 10–20 cm in diameter at 20–25 °C and were >6 weeks old when flowers were collected for analyses.

Hand-pollination experiment

In order to standardize the stages of PACC, five genetic individuals of *L. filicaulis* were tagged for both control and treatment (hand pollination), monitored daily for colour change and photographed to capture distinct colour stages. Three flowers were measured per plant for both control and treatment. Hand pollination was conducted 1 d after anthesis (flower opening) to ensure the keel petals had separated. Pollen was transferred between flowers using an implement of a Victorinox SwissTool™ (unpointed blade).

The rate of progression though these stages was modelled using the lme4 package (Bates *et al*., 2015) in R version 3.2.2 (R Development Core Team, 2008). Data were visualized using the ggplot2 package (Wickham, 2009) in R. The R scripts used in this analysis are available at github.com/mannfred/Lotus.

Transcriptome sequencing

The entire corollas (dorsal, lateral and ventral petals) of *L. filicaulis* and *L. sessilifolius* were separated from the rest of the flower at the same developmental stage (Stage 13 of anthesis, Ojeda *et al*., 2012a), but with two different colour stages: (1) flowers at anthesis with a yellow colour; and (2) flowers initiating flower colour change to red. At least six unpollinated flowers of each type, yellow and red, were collected from one individual. These *Lotus* species undergo PACC independently of visitation by putative pollinators. Petals of each colour were removed from the flower and pooled to represent the phenotype across the entire individual. Flower petals were immediately placed on liquid nitrogen after collection and stored at −80 °C

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until RNA extraction. RNA was extracted using the PureLink™ Plant RNA Reagent from Invitrogen following the manufacturer's protocol. RNA quantity and quality were determined using an Invitrogen Qubit® 2·0 (Life Technologies) and with an Agilent 2100 Bioanalyzer, respectively. Samples with an RNA integrity number (RIN) of $\geq$7 were used for library construction. Paired end-RNA libraries (Supplementary Data Tables S38 and S39) were prepared for each individual sample with the Illumina kit according to the manufacturer's protocol. All samples were sequenced (100 base-pair reads) in one lane of an Illumina Hi-Seq 2000 at the NextGen Sequencing Facility at the Biodiversity Research Centre, University of British Columbia (UBC).

Sequence analysis

The utility of aligning RNA-Seq data from *L. filicaulis* and *L. sessilifolius* to *L. japonicus* has not previously been assessed. Therefore, we compared two methods to discover differentially expressed genes relevant to PACC. In the first method, RNA-Seq reads were aligned to the reference genome of a closely related model organism, *L. japonicus*. In the second method, RNA-Seq reads were assembled into contigs (putative genes) and aligned to a reference transcriptome that was assembled *de novo* from the reads themselves. All scripts used in the analyses are available at github.com/mannfred/Lotus. Contig sequences (as listed in Tables 3 and 4) are deposited in GenBank (accession numbers KY865588–KY865608).

TopHat alignment

RNA-Seq reads from *L. filicaulis* and *L. sessilifolius* were analysed by TopHat. FASTQC Galaxy v0.63 (Andrews, 2010) was executed on a public Galaxy server (Goecks *et al*., 2010) to check the quality of reads and to identify sequencing artefacts. Sequencing reads were mapped to the *L. japonicus* genome release 2·5 (Sato *et al*., 2008) using TopHat2 Galaxy v0.9 (Kim *et al*., 2013). Sequence Alignment Map (.sam) files were processed by HTSeq (Anders *et al*., 2015) to count the number of RNA-Seq reads that mapped to each TopHat feature (gene), and differential expression (DE) was calculated using edgeR (Robinson *et al*., 2010). Raw counts per gene were used instead of FPKM (fragments per kilobase per million)-transformed counts because edgeR incorporates its own dispersion-normalization procedure in its DE calculations (Robinson *et al*., 2010). EdgeR results were processed using a set of custom R scripts to filter significant results [Benjamini–Hochberg FDR (false discovery rate) $<$0·05 (Benjamini and Hochberg, 1995)] and differences in fold change.

The DE genes were analysed for ontology term enrichment [Yekutieli FDR $<$0·05 (Yekutieli and Benjamini, 1999)] using the Singular Enrichment Analysis tool with the *L. japonicus* 2·5 genome reference in AgriGO (Du *et al*., 2010). Because the functional annotation of the *L. japonicus* genome is ongoing, genes associated with enriched gene ontology (GO) categories were annotated for putative function by BLASTing the *Glycine max* CDS library (Williams 82 Assembly 2 version 1; Schmutz *et al*., 2010) sourced from Phytozome v10.3 (Goodstein *et al*., 2012)

STAR alignment

RNA-Seq reads from both *L. filicaulis* and *L. sessilifolius* were mapped to the *L. japonicus* genome Kazusa version 2.5 (Sato *et al*., 2008) using STAR v2.4 (Dobin *et al*., 2013). Successfully mapped transcripts were assembled and expression was estimated using RSEM v1.2.19 (Li and Dewey, 2011). Differential expression of the yellow and red flower transcriptomes was calculated using the same methods as above.

De novo *assembly by Trinity*

Sequenced Illumina reads from *L. filicaulis* and *L. sessilifolius* were assembled *de novo* into contigs in Trinity v2.0.6 (Grabherr *et al*., 2011). Using assembled contigs as a reference transcriptome, contigs were measured for expression using Trinity's built-in RSEM tool (Li and Dewey, 2011) and DE was calculated as above. The DE contigs were then identified by BLASTn (Altschul *et al*., 1997) as their top-scoring *L. japonicus* homologue (expectation value of 1e−4) using the *L. japonicus* v2.5 CDS library sourced from Kazusa (Sato *et al*., 2008). Ontology term enrichment and annotation of associated genes were analysed as above.

Nucleotide alignments

Trinity contigs BLASTing to *L. japonicus* genes of interest were investigated further by BLASTing the Legume IP database (Li *et al*., 2011) for *G. max* (v2.0) (Schmutz *et al*., 2010), *Medicago truncatula* (Mt4.0v1) (Young *et al*., 2011) and an outgroup, *Arabidopsis thaliana* (TAIR10) (Lamesch *et al*., 2011), to further identify homologous genes. Trinity contigs were trimmed before and after putative start and stop codons, which were identified by pairwise sequence alignment of their *L. japonicus* homologue using EMBOSS Needle (Rice *et al*., 2000). Coding sequences of putative homologues were compiled along with the relevant trimmed Trinity contig into a .fasta file and translated to amino acid sequences using TranslatorX (Abascal *et al*., 2010). Multiple sequence alignment was performed using the T-Coffee (Notredame *et al*., 2000) option in TranslatorX. The phylogenetic relationship of a given Trinity contig to its relatives was visualized using RAxML v8.2 (Stamatakis, 2014) in Mesquite (Maddison and Maddison, 2015).

Gene family composition

Trinity (*de novo* assembly) sequences were BLASTed (1e−4) against the *L. japonicus* CDS library at LegumeIP to detect gene duplicates – specifically those not expressed at the time of sampling (this approach assumes no new gene duplications have occurred between *L. japonicus* and our two study species). BLAST results sharing the same annotation (i.e. gene name) as the top alignment were considered to be a part of the same gene family.

RESULTS

Pollination accelerates colour change in L. filicaulis

Four distinct colour stages of PACC were observed: (1) entirely yellow; (2) blush of orange, typically at the keel; (3) systemic

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orange coloration across the corolla; and (4) red, onset typically at the keel (Fig. 3). The general linear model to test the effects of time (d, X_1) and treatment (X_2) on colour (Y) was:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 \times X_2$$

where β_i, $i = \{1,2,3\}$ are constants. Among-plant standard deviation was effectively zero (s =2.664 e−05) and was omitted as a random effect.

The number of days from anthesis was a significant predictor of colour (d.f. = 1,39; P =1·69 e−09), as well as the effect of treatment through time (d.f. =1,39; P =5·06 e−04).

The rate of progression through the four stages of PACC in the hand-pollinated treatment is over twice that of the control (see Fig. 3 caption).

Illumina HiSeq library

A total of 12.5 million and 11.9 million .fastq reads were sequenced from the yellow-stage and red-stage RNA-Seq libraries of *L. filicaulis*, respectively. The GC content was 46 % for the forward yellow-stage and red-stage reads and 45 % for the reverse reads.

Similarly, 6.7 million and 12.0 million .fastq reads were sequenced from the yellow-stage and red-stage RNA-Seq libraries of *L. sessilifolius*, respectively. The GC content was 45 % for the forward yellow-stage and red-stage reads and 44 % for the reverse reads.

De novo *assembly of non-model organism RNA reads outperforms alignment of reads to a reference genome of a closely related species*

Two methods were used to discover differential expression in PACC: (1) direct mapping of reads to the *L. japonicus* genome using two different splice junction mappers; and (2) *de novo* assembly of reads to contigs followed by BLASTing these contigs to an *L. japonicus* CDS library. The evaluation of the two approaches is summarized in Tables 1 and 2. Direct alignment by STAR and TopHat achieved low mapping rates due to unsuccessful heterologous mapping from our study species to *L. japonicus*. However, the *de novo* assembly protocol achieved a high rate of gene identification, and produced independent contigs that could be verified for identity and biological relevance by amino acid alignment and phylogenetic methods.

Trinity contigs of interest were found to align with homologous genes of related legumes, and their known phylogenetic relationship was successfully predicted by these alignments (Supplementary Data Figs S1–S59).

The cyanidin pathway is upregulated in the red stage of PACC in L. filicaulis *and* L. sessilifolius

Regardless of the bioinformatics protocol, upregulation of numerous genes in the anthocyanin biosynthetic pathway (ABP) was detected in the red stage of PACC in both *Lotus* species examined (Tables 3 and 4, Figs 4 and 5). Except for *ANTHOCYANIN SYNTHASE* and *FLAVONOID 3′-HYDROXYLASE* (both apparently single-copy), gene duplicates or multiple gene families were identified in the *Lotus japonicus* genome for each of the genes of the ABP in both species (Figs 4 and 5). Numerous transcription factors related to flavonoid biosynthesis were detected in the transcriptomes of both *Lotus* species (Supplementary Data Tables S13–S15, S29–31). Of these, we have focused the discussion on *PAP1*, *TT8* and *TTG1* (Figures 4 and 5). As a comparison the same analysis was carried out for the lignin biosynthetic pathway (LBP), which is not expected to be associated with PACC. There is no consistent upregulation of LBP genes during PACC. Generally, LBP genes are not expressed, downregulated during PACC or not differentially expressed (Figs 4 and 5).

The carotenoid pathway is upregulated in the yellow stage of PACC in L. filicaulis *and* L. sessilifolius

Genes annotated to the carotenoid biosynthetic process (GO:0016117) were significantly upregulated at the yellow stage (i.e. downregulated at the red stage of PACC) in both *Lotus* species (Supplementary Data Tables S35–S37). The core carotenoid pathway is represented, including the two major branches producing zeaxanthin and lutein (Figs 6 and 7).

Differences between L. filicaulis *and* L. sessilifolius

A congeneric comparison of single transcriptomes is limited in precision; nonetheless, marked differences between these *Lotus* species are considered to be testable models. Although the cyanidin pathway is upregulated during PACC in both *Lotus* species, there are differences in the composition of genes expressed, specifically those genes that are a part of a gene family. For example, *CHALCONE SYNTHASE* (*CHS*) is active in both species but the gene family members expressed in *L. filicaulis* and *L. sessilifolius* are disjoint sets. Similar differences are observed in the expression of *DFR*. However, it is notable that of the two *F3H* duplicates known for *L. japonicus*, only one is implicated in PACC for the two *Lotus* species studied. The expression of transcription factors and regulators contributes to numerous other differences between PACC in *L. filicaulis* and *L. sessilifolius*. For example, we detect that *PAP1* and *TT8* are differentially expressed at the yellow stage of PACC in *L. filicaulis*, but in *L. sessilifolius PAP1* was not detected and

TABLE 1. *Comparison of protocols used to detect differential expression of RNA in* L. filicaulis

Analysis type (mapping reference)	Reads mapped, yellow stage	Reads mapped, red stage	DE genes yellow stage ↑	DE genes, red stage ↑	Enriched GO terms, yellow stage	Enriched GO, terms red stage
TopHat (*L. japonicus* genome)	8 538 623	7 243 178	1419	962	107	84
STAR (*L. japonicus* genome)	4 334 187	3 566 554	1398	958	128	97
Trinity (*L. filicaulis* transcriptome)	14 025 763	13 167 862	3287	1244	112	44

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TABLE 2. *Comparison of protocols used to detect differential expression of RNA in* L. sessilifolius

Analysis type (mapping reference)	Reads mapped, yellow stage	Reads mapped, red stage	DE genes, yellow stage ↑	DE genes, red stage ↑	Enriched GO terms, yellow stage	Enriched GO terms, red stage
TopHat (*L. japonicus* genome)	1 333 258	1 855 091	394	632	122	95
STAR (*L. japonicus* genome)	1 272 184	1 976 009	610	954	151	120
Trinity (*L. sessilifolius* transcriptome)	13 085 868	23 373 724	1175	1468	100	57

TABLE 3. *Selected genes for proteins involved in flavonoid biosynthesis in* L. filicaulis. *The first column lists* de novo *Trinity contigs. The second column lists the gene names produced by aligning contigs to the* Glycine max *CDS library (abbreviations are defined in the legend of Fig. 4). The third and fourth columns list raw counts per gene at each colour stage, followed by the normalized log_2 fold change. Sequences for the Trinity contigs in this table are available in GenBank (accession numbers KY865588–KY865608)*

Trinity contig	*Glycine max* homologue	Counts per gene (yellow stage)	Counts per gene (red stage)	Normalized log_2 fold change (red/yellow)
TR19889|c3_g1	Glyma.01g43880·1 *CHS*	1385	19 064	−4·1392
TR48518|c0_g1	Glyma.11g011500·1 *CHS*	77	430	−2·8356
TR19889|c2_g1	Glyma.01g228700·1 *CHS*	17	207	−3·9514
TR43354|c0_g1	Glyma.20g241700·1 *CHI*	1354	551	−1·6533
TR38960|c0_g1	Glyma02g05450·1 *F3H*	1830	6123	−2·0988
TR52191|c0_g1	Glyma.06g202300·1 *F3'H*	1586	15638	−3·6580
TR29586|c0_g1	Glyma14g07940·1 *DFR*	148	10163	−6·4567
TR50049|c0_g1	Glyma01g42350·1 *ANS*	1010	14783	−4·2278
TR32422|c0_g1	Glyma.02g147800·1 *TT8*	131	0	9·8676
TR13738|c0_g3	Glyma04g40610·1 *TTG1*	706	252	1·1293
TR11002|c0_g1	Glyma14g06750·1 *PAP1*	475	54	2·7778

TT8 was expressed equally through PACC. Additionally, we detected expression of *TTG1* at both stages of PACC in both *Lotus* species (Tables 3 and 4, Figs 4 and 5).

Finally, we draw attention to the presence of *FLAVONOL SYNTHASE* (*FLS*) in *L. sessilifolius* (Table 4, Fig. 5). We detect an upregulation of FLS during the yellow stage of PACC in this species, and an absence of activity of this gene in *L. filicaulis*.

DISCUSSION

Significance of pollination as a trigger for PACC

We show that PACC is accelerated by pollination in *L. filicaulis* (Fig. 3); these measurements support those made of *A. glaber* (Jones and Cruzan, 1999). This is suggestive that PACC is a trait that has evolved via interaction with pollinators rather than an incidental characteristic of the ageing process with no fitness consequences. This is unsurprising as many floral traits are indeed driven by pollinators (Schiestl and Johnson, 2013) and it is consistent with this trait being a widespread evolutionary convergence (Weiss, 1995). Viewing PACC as an evolved adaptive trait raises the question of the precise adaptive function that it confers. Brito *et al*. (2015) suggest that the retention of flowers increases attraction of insect pollinators at long range, and then at short range increases foraging efficiency by signalling flower quality to pollinators.

The demonstration of a pollination trigger for PACC also raises the question of what the precise nature of the trigger is. Pollination involves physical movement of the petals of the flower (thigmostimulus), deposition of pollen on the stigma, subsequent growth of pollen tubes and fertilization of the ovules. All these processes have physiological consequences and could be transduced into signals leading to PACC. Further experimental work is required to dissect the events of pollination that lead to the acceleration of PACC. Even without pollination, colour change eventually occurs. This suggests that there is an additional, late-acting or fail-safe trigger for the onset of PACC, one that presumably responds either to late-acting autogamy or to some aspect of floral ageing.

De novo *assembly is preferred for RNA-Seq data without a reference genome*

The two RNA-Seq aligners used in our study, STAR and TopHat, performed similarly in aligning sequenced reads to the

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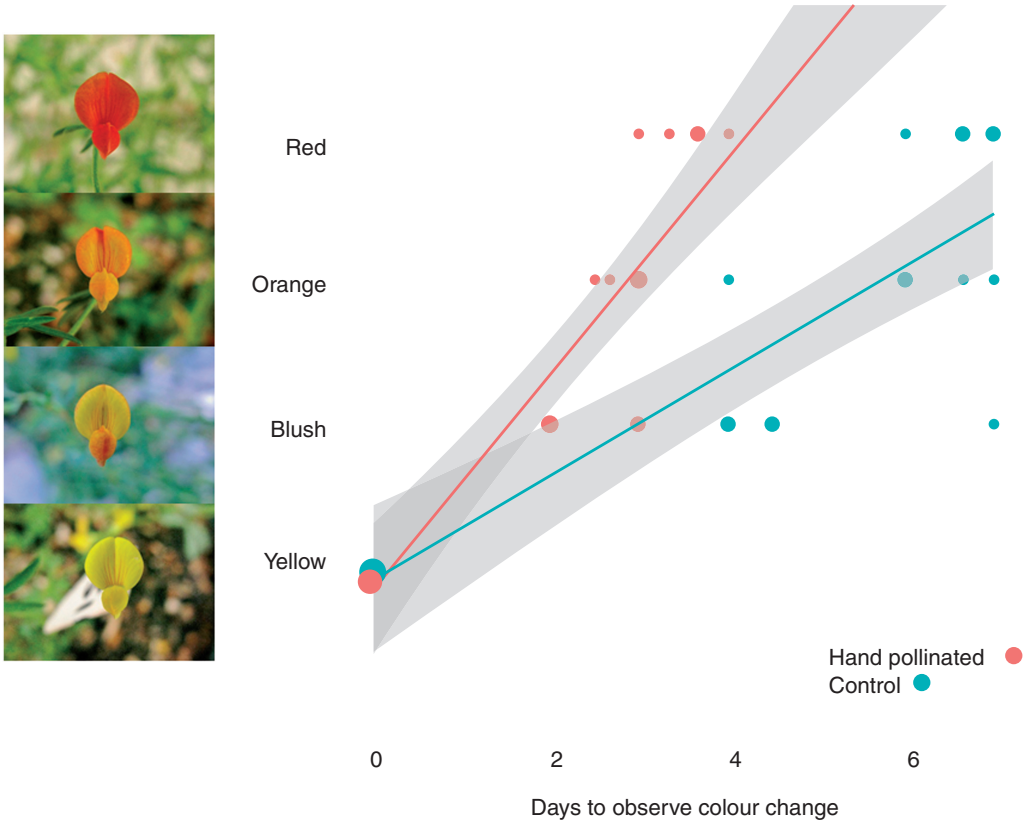

FIG. 3. (Left panel) Standardization of flower colour using photographs of four distinct stages: anthesis ('Yellow', stage 0); onset of keel colouration ('Blush', stage 1); systemic orange across the corolla ('Orange', stage 2); and full coloration ('Red', stage 3). (Right panel) Graph showing timing of stages in the hand-pollinated treatment [orange line, colour stage = 0·739 (d) – 0·121] and control (unpollinated) [turquoise line, colour stage = 0·358 (d) – 0·059]. Intercepts were not fixed (anthesis) to maintain zero-mean residuals. Each point represents the mean number of days to one of four stages (mean of three replicates per treatment per individual plant). The size of points is proportional to the amount of over-plotted data.

genome of a sister species. Our results are consistent with a recently published comparison of 11 commonly used alignment protocols (Engström *et al*., 2013). STAR 1-pass uses gene annotation information to identify exon splice junctions before aligning to the reference genome. TopHat also uses an annotation file to identify junctions, and furthermore uses this information to align reads directly against annotated transcripts. Therefore, both alignment protocols require a well-annotated genome, which currently does not exist for *L. filicaulis* or *L. sessilifolius*. Aligning *L. filicaulis* and *L. sessilifolius* reads to the genome of *L. japonicus* led to a substantial loss of data as the majority of reads remained unmapped. However, the number of *L. filicaulis* reads mapped to *L. japonicus* outperformed the number of mapped *L. sessilifolius* reads when considering the same protocol. This underscores the importance of phylogenetic relatedness when using a model genome to study a congener.

Our results strongly support the use of a *de novo* assembly of RNA-Seq reads into a transcriptome (then mapping reads back onto the transcriptome) as opposed to attempts at cross-species mapping in *Lotus*. Contigs may then be identified by reciprocal BLAST (with putative genes checked by amino acid alignment) against the most closely related species with an annotated genome.

PACC is associated with the wholesale upregulation of the anthocyanin pathway

Ojeda *et al*. (2013) previously studied gene expression of some flavonoid biosynthesis genes involved in PACC in *L. filicaulis* and *L. sessilifolius* by PCR. Our results support their findings and present several new perspectives from which PACC should be considered. With the exception of *CHALCONE ISOMERASE*, at least one copy of each gene in the flavonoid biosynthetic pathway leading to cyanidin production is differentially expressed (increased) at the red stage of PACC (Figs 4 and 5). An alternative scenario might be that most of these genes might already be expressed at the yellow stage with the exception of one critical enzyme without which pigment cannot be made. Under this scheme the upregulation of a single enzyme would be enough to initiate colour change. Our results make it clear that this is not the case. Instead nearly the entire pathway is upregulated. Except for *CHALCONE ISOMERASE*, all genes in the pathway have relatively low expression at anthesis and increase dramatically at the PACC stage (Tables 3 and 4, Figs 4 and 5). This implies that the genes in the pathway are co-regulated as a unit during PACC, possibly by a combination of transcription factors.

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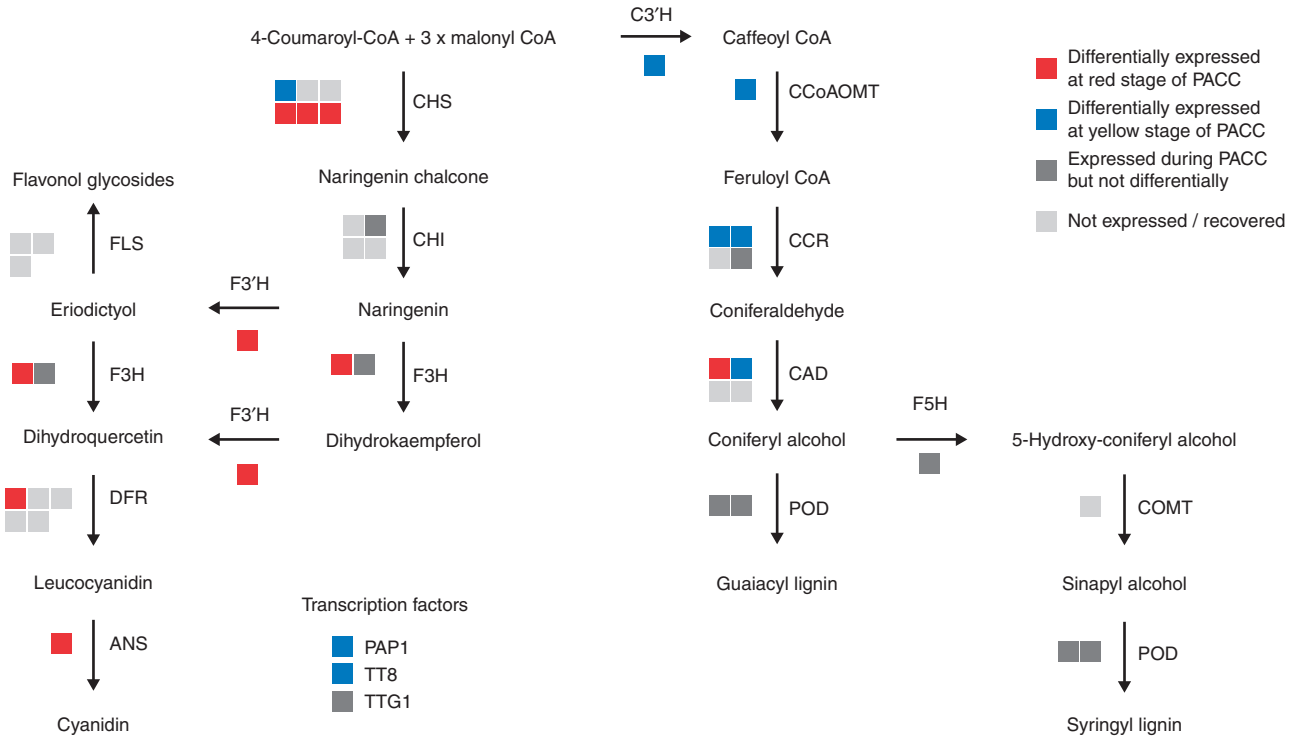

FIG. 4. Graphical representation of the differential expression of genes (Trinity contigs) involved in flavonoid and lignin biosynthesis throughout PACC in *Lotus filicaulis*. The boxes represent genes as annotated in the *Lotus japonicus* genome; light grey boxes represent genes that were not found in the *L. filicaulis* transcriptome (gene may be absent or may have been undetected). Expression of putative orthologues in *L. filicaulis* are indicated by colour (red, upregulated at red stage; blue, upregulated at yellow stage; dark grey, not differentially expressed). 4-Coumaroyl-CoA and malonyl CoA are derivatives of phenylalanine and acetatepyruvate, respectively. Flavonoid pathway: CHS, chalcone synthase; CHI, chalcone isomerase; F3′H, flavonoid 3′-hydroxylase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase. Lignin pathway: C3′H, coumaroyl shikimate 3′-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; POD, peroxidase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid *O*-methyltransferase.

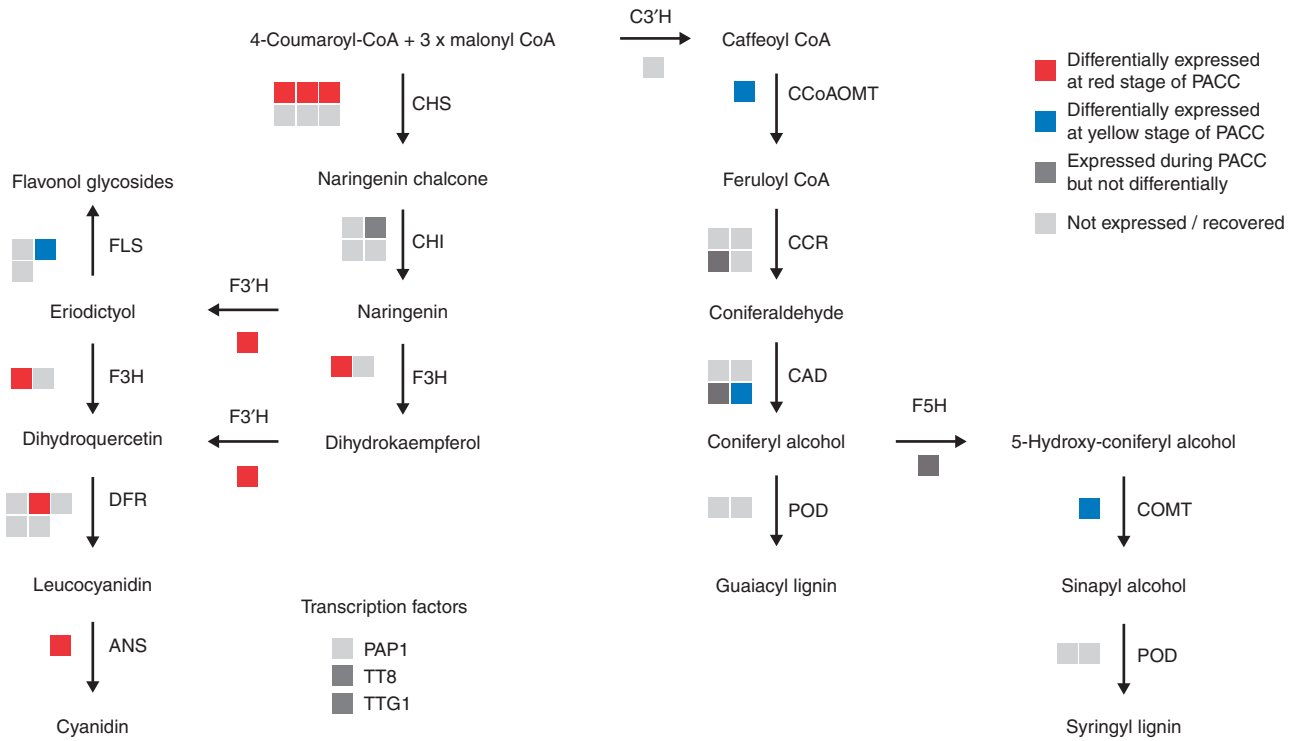

FIG. 5. Graphical representation of the differential expression of genes (Trinity contigs) involved in flavonoid and lignin biosynthesis throughout PACC in *Lotus sessilifolius*. Conventions and acronyms are as defined in the legend of Fig. 4.

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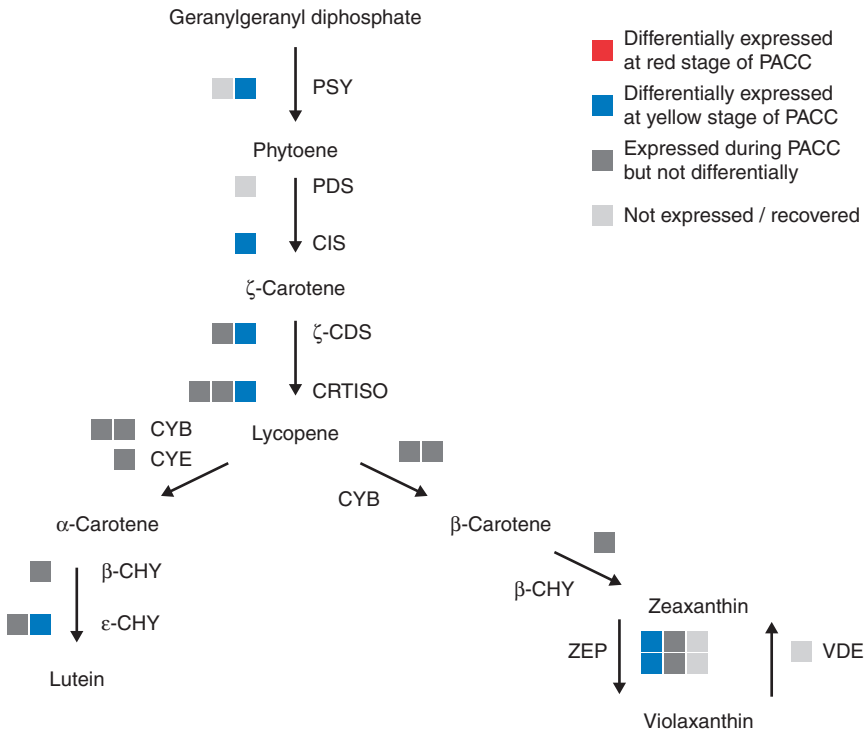

FIG. 6. Graphical representation of the differential expression of genes (Trinity contigs) involved in carotenoid biosynthesis throughout PACC in *Lotus filicaulis*. Boxes represent genes as annotated in the *Lotus japonicus* genome; light grey boxes represent genes that were not found in the *L. filicaulis* transcriptome (gene may be absent or may have been undetected). Expression of putative orthologues in *L. filicaulis* are indicated by colour (red, upregulated at red stage; blue, upregulated at yellow stage; dark grey, not differentially expressed). Geranylgeranyl diphosphate is a derivative of pyruvate and glyceraldehyde 3-phosphate. PSY, phytoene synthase; PDS, phytoene desaturase; CIS, carotene isomerase; ζ-CDS, ζ-carotene desaturase; CRTISO, prolycopene isomerase; CYB, β-lycopene cyclase; CYE, ε-lycopene cyclase; β-CHY, β-carotene hydroxylase; ε-CHY, ε-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase.

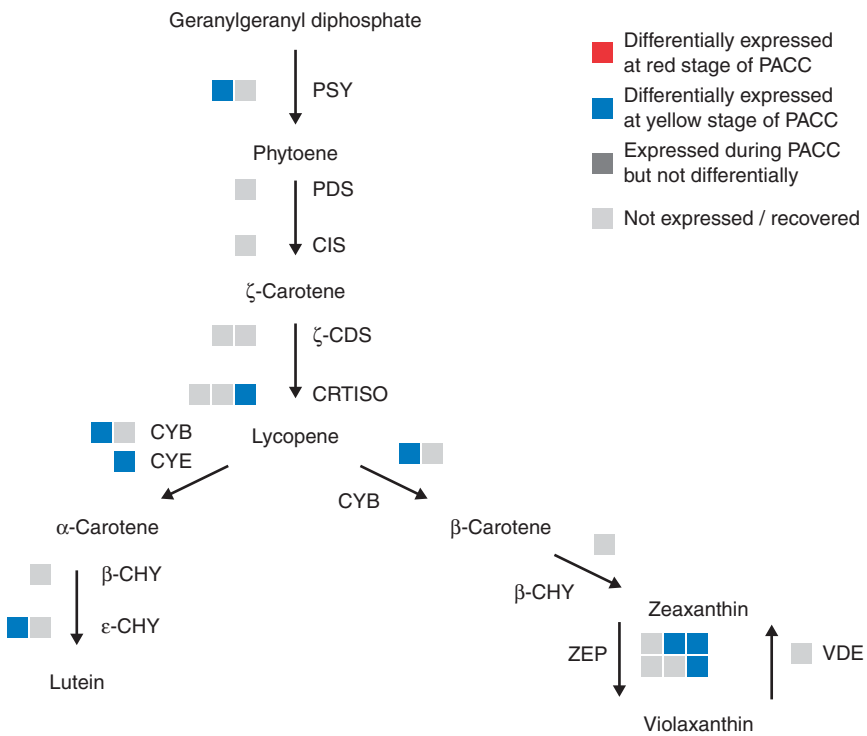

FIG. 7. Graphical representation of the differential expression of genes (Trinity contigs) involved in carotenoid biosynthesis throughout PACC in *Lotus sessilifolius*. Conventions and acronyms are as defined in the legend of Fig. 6.

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TABLE 4. *Selected genes for proteins involved in flavonoid biosynthesis in* L. sessilifolius. *See Table 3 title for column descriptions*

Trinity contig	*Glycine max* homologue	Counts per gene (yellow stage)	Counts per gene (red stage)	Normalized $\log_2$ fold change (red/yellow)
TRINITY_DN5 4168_c0_g2	Glyma.11G011500·1 *CHS*	65	4382	−6·1743
TRINITY_DN5 4168_c0_g1	Glyma.11G011500·1 *CHS*	17	1134	−6·1510
TRINITY_DN5 2652_c1_g1	Glyma.19G105100·1 *CHS*	145	660	−2·2875
TRINITY_DN15851_c0_g1	Glyma.20G241700·1 *CHI*	247	430	−0·9016
TRINITY_DN5 2390_c0_g1	Glyma.02G048400·1 *F3H*	276	1893	−2·8794
TRINITY_DN5 2780_c0_g1	Glyma.06G202300·1 *F3'H*	7	2073	−8·2859
TRINITY_DN5 2789_c0_g1	Glyma.17G252200·1 *DFR*	19	434	−4·6063
TRINITY_DN5 3094_c0_g1	Glyma.11G027700·1 *ANS*	30	3761	−7·0659
TRINITY_DN3 9800_c0_g1	Glyma.13G082300·1 *FLS*	1601	112	3·7338
TRINITY_DN54056_c0_g2	Glyma.02G147800·1 *TT8*	182	151	0·1671
TRINITY_DN48324_c0_g1	Glyma04g40610·1 *TTG1*	570	493	0·3114

PACC is associated with highly specific upregulation of members of gene families

Many of the enzymes involved are coded by multiple genes in gene families. An example is *DFR*, which has numerous copies (*DFR1–DFR5*) in *L. japonicus* (Shimada *et al*., 2005). However, during PACC only one paralogue is upregulated. Other examples are available from inspection of Figs 4 and 5. In these cases, the upregulation of specific copies, rather than all copies of a particular enzyme gene, seems to drive PACC. Some gene copies are apparently not expressed at either stage, but may be involved in tissue-specific expression elsewhere. It is worth noting that different gene copies within the *DFR* and *CHS* families are expressed between *L. filicaulis* and *L. sessilifolius*. This is also the case for several gene families of the carotenoid pathway (Figs 6 and 7). This lends support to the convergent evolution of PACC between *L. filicaulis* and *L. sessilifolius*, as hypothesized by Ojeda *et al*. (2013) (Fig. 2). However, it remains possible that, regardless of differences in the upregulation of gene copies, some general aspect of the flavonoid pathway (e.g. how it is regulated) is a developmental pre-pattern for PACC that is common to *Lotus*. In this case, the apparent convergent evolution of PACC could be considered a 'latent homology' (Nagy *et al*., 2014).

Because anthocyanins (a class of flavonoids) are highly versatile and serve a wide range of functions (Kong, 2003), it may be that gene duplication is key to the evolution of novel adaptations involving anthocyanins, including PACC. Neo-functionalization, where directional selection leads to novel function following a gene duplication event, may explain why only certain copies of flavonoid biosynthesis genes were actively transcribed during PACC. Alternatively the association of PACC with duplicated genes might be explained by escape from adaptive conflict (Des Marais and Rausher, 2008). In this scenario a single-copy gene is adapted to simultaneously perform its ancestral function while performing some novel function, but is constrained in its ability to improve due to antagonistic pleiotropy. Following gene duplication, both ancestral and novel functions have the potential to be better adapted. Des Marais and Rausher (2008) demonstrated that adaptive changes in the *DFR* gene of preduplication and postduplication plant species are best explained as a means of escape from adaptive conflict. In order to determine whether PACC is a result of gene duplication, evidence for the ancestral and novel functions of flavonoid biosynthesis genes would need to be studied in *L. filicaulis*, *L. sessilifolius* and their relatives. In the case of *L. japonicus*, various duplicates of *DFR* differ in their responses to stress and organ specificity (Yoshida *et al*., 2010), but whether any such gene copies are functionally divergent in our study species and whether any of these copies are flower-specific remain unstudied.

Upregulation of flavonoid biosynthesis may be antagonistic to other phenylpropanoid pathways

Expression of genes in the LBP suggests that a shift from the lignin branch of the phenylpropanoid pathway to the flavonoid branch may occur over the course of PACC (Figs 4 and 5). Transcriptome and metabolite profiling of *Fragaria* × *ananassa* has demonstrated a metabolic interaction between flavonoid and lignin biosynthesis in strawberry fruit (Ring *et al*., 2013). Lignin biosynthesis competes for the same upstream substrate (coumaroyl-CoA) as flavonoid biosynthesis and, as would be expected under constant substrate availability, a shift from lignin production to flavonoid production can be measured in anthocyanin-rich tissues (Ring *et al*., 2013). Therefore it is plausible that in *L. filicaulis* and *L. sessilifolius* a drop in substrate availability may result in the downregulation of LBP genes observed during PACC.

Alternatively, expression of LBP genes may be reduced following flowering, regardless of PACC, simply due to developmental changes associated with ageing.

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Carotenoid biosynthesis is downregulated during PACC

Carotenoids are widespread in the yellow to orange flowers of numerous insect-pollinated taxa, and likely play a role in the detectability of flowers by pollinators (Cronk and Ojeda, 2008; Tanaka and Ohmiya, 2008;). Consequently, the biosynthesis of carotenoids is generally well understood (Zhu *et al*., 2010).

We detected the upregulation of the genes controlling the core carotenoid pathway at the yellow stage of PACC in both *Lotus* species – this is expected given the definitively yellow appearance of the flowers of both species at anthesis. To the extent that flavonoid biosynthesis may be crucial to the red stage of PACC, the coincident downregulation of carotenoid biosynthesis genes may be important in accentuating colour change. Alternatively, the downregulation of carotenoids may represent a senescence-related process separate from flavonoid production.

Transcription factors regulating flavonoid biosynthesis

The MYB, bHLH and WD repeat (WDR) proteins are known to be regulators of the flavonoid biosynthetic process in several plant taxa and can interact to form MBW complexes (recently reviewed in Xu *et al*., 2015). Both *Lotus* transcriptomes recovered sequences for transcription factors known to be important regulators of flavonoid biosynthesis: in *L. filicaulis* we detected the differential expression of a potential orthologue of *PAP1* (R2R3-MYB) and *TT8* (bHLH-042) at the yellow stage of PACC, while in *L. sessilifolius* we did not detect *PAP1*, and *TT8* was not differentially expressed. In both *Lotus* species *TTG1* (WDR) was expressed equally at both stages of PACC.

In *Arabidopsis*, *PAP1* and *TT8* interact to regulate the expression of *DFR* (Zimmerman *et al*., 2004), and similar MBW interactions in the flavonoid pathway have been documented (Xu *et al*., 2015). A possible explanation for the activity of *PAP1* and *TT8* in *L. filicaulis* is that these products are activators of the ABP, and therefore need to be synthesized ahead of the structural genes. However, this counters the current consensus that *TT8* complexes generally control the expression of downstream genes (i.e. 'late' biosynthetic genes) during flavonoid biosynthesis (Baudry *et al*., 2006; Xu *et al*., 2015). In *Arabidopsis*, anthocyanin and proanthocyanin biosynthesis is *TTG1*-dependent, and forms MBWs with *TT8* and *PAP1* (Baudry *et al*., 2004; Dubos *et al*., 2010). Because of the modular nature of MBWs, especially versatile transcription factors (e.g. *TTG1*) may be active at many stages of flower development; thus we observe that the expression of *TTG1* is constant throughout the PACC process.

The role of FLS in PACC in L. sessilifolius

FLS forms flavonols (quercetin and kaempferol) from dihydroflavonols, the same substrate used by *DFR* to produce cyanidin. *FLS* has been shown to be antagonistic to anthocyanin accumulation, which increases in *FLS* knockouts (Lee *et al*., 2016). Ojeda *et al*. (2013) demonstrated that the transition from bee- to bird-pollinated *Lotus* species involves the increased biosynthesis of anthocyanins at the expense of flavonol production, and that pre-colour change (yellow-flowered) *Lotus* species from the Canary Islands contain comparatively more flavonols (mostly isorhamnetin) than anthocyanins. Flavonols are generally colourless to the human eye but absorb strongly in the UV region. Despite this, Ojeda *et al*. (2013) demonstrated a reduction in UV reflectance of the petals following PACC (Ojeda *et al*., 2013). Whatever the mechanism, a shift from flavonol to cyanidin production may affect the detectability of the flowers by pollinators. It is of interest that *L. sessilifolius* and *L. filicaulis* differ markedly in the behaviour of *FLS*.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

Supplementary data are available online at www.oxfordjournals.org and consist of the following: Tables S1–S3: *L. filicaulis* TopHat results: genes for enzymes annotated to GO:0009813 (flavonoid biosynthetic process) and GO:0009809 (lignin biosynthetic process). Tables S4–S6: *L. filicaulis* STAR results: genes for enzymes annotated to GO:0009813 (flavonoid biosynthetic process) and GO:0009809 (lignin biosynthetic process). Tables S7–S12: *L. filicaulis* Trinity results; genes for enzymes annotated to GO:0009813 (flavonoid biosynthetic process) and GO:0009809 (lignin biosynthetic process). Table S13: *L. sessilifolius* TopHat results: genes for enzymes annotated to GO:0009813 (flavonoid biosynthetic process). Tables S14–S16: *L. sessilifolius* STAR results: genes for enzymes annotated to GO:0009813 (flavonoid biosynthetic process) and GO:0009809 (lignin biosynthetic process). Tables S17–S22: *L. sessilifolius* Trinity results: genes for enzymes annotated to GO:0009813 (flavonoid biosynthetic process) and GO:0009809 (lignin biosynthetic process). Table S23: *L. filicaulis* yellow stage Trinity results: genes for enzymes annotated to GO:00016117 (carotenoid biosynthetic process). Table S24: *L. filicaulis* similarly expressed Trinity results: genes for enzymes annotated to GO:00016117 (carotenoid biosynthetic process). Table S25: *L. sessilifolius* yellow stage Trinity results: genes for enzymes annotated to GO:00016117 (carotenoid biosynthetic process). Table S26: next-generation library construction for *L. filicaulis*. Table S27: next-generation library construction for *L. sessilifolius*. Table S28: function of flavonoid biosynthesis genes of cited in Tables 3 and 4. Figures S1–S17: amino acid alignments of putative carotenoid biosynthetic genes in *L. filicaulis* and related

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legumes. Figures S18–S30: amino acid alignments of putative flavonoid biosynthetic genes in *L. filicaulis*-related legumes. Figures S31–S40: amino acid alignments of putative lignin biosynthetic genes in *L. filicaulis* and related legumes. Figures S41–S46: amino acid alignments of putative carotenoid biosynthetic genes in *L. sessilifolius* and related legumes. Figures S47–S54: amino acid alignments of putative flavonoid biosynthetic genes in *L. sessilifolius* and related legumes. Figures S55–S59: amino acid alignments of putative lignin biosynthetic genes in *L. sessilifolius* and related legumes. Figures S60–S67: ReviGO Heatmaps of TopHat and STAR analyses for *L. filicaulis* and *L. sessilifolius*. Figures S68–S73: ReviGO Heatmaps of Trinity analysis for *L. filicaulis* and *L. sessilifolius*.

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