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COMPARATIVE TRANSCRIPTOME ANALYSIS OF TWO CLOSELY RELATED *BEGONIA* SPECIES REVEALS DIVERGENT PATTERNS IN KEY LIGHT-REGULATED PATHWAYS

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Begonia is one of the most diverse angiosperm genera, comprising more than 2000 described species. The wide morphological and ecological range represented in the genus makes it a good model for studying the generation and maintenance of diversity. Previous research has shown that strong population structure, poor seed dispersal, and high levels of drift contribute to high rates of speciation and morphological variation. In the present study, we used transcriptomics to compare two closely related but morphologically and ecologically divergent species, *Begonia conchifolia* A.Dietr. and *B. plebeja* Liebm., to identify genes putatively involved in ecological divergence. Using publicly available multitissue RNA-seq data, we asked what genetic pathways show species-specific patterns of divergence between our two study species. The results of differential expression and gene ontology enrichment analyses showed species-specific enrichment of light-regulated functions in *Begonia plebeja*. Concomitant enrichment of ethylene and jasmonate pathways in *Begonia plebeja* indicate an increased shade avoidance response, suggesting that light availability may be a key factor in the divergent adaptation of *B. conchifolia* and *B. plebeja*.

Keywords. *Begonia*, comparative transcriptomics, diversification, light regulation, shade avoidance, transcriptomics.
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Introduction

Understanding the evolutionary processes governing adaptation and speciation is fundamental to identifying the drivers of diversification and can inform biodiversity and conservation studies (Conover *et al.*, 2006; Seehausen, 2006; Wilcox *et al.*, 2019). Selection can drive divergent adaptation in populations that are presented with ecological opportunities in the form or new or underused niches (Fjeldså *et al.*, 2018), targeting ecologically relevant genes and regulatory pathways and driving differentiation across other, neutral genomic regions (Feder & Nosil, 2010), which can reinforce species barriers post divergence by the reduction of gene flow (Nosil *et al.*, 2009). Identification of genes underlying adaptation to new niches is necessary to understand the biotic and abiotic factors that drive adaptive change (Fischer *et al.*, 2013; Yeaman *et al.*, 2016), and in

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some cases can be used to predict the adaptive potential of genetic changes (Turner *et al.*, 2010; Hancock *et al.*, 2011). The study of functional classes of genes involved in ecological adaptation is, therefore, key to understanding environmental factors responsible for driving adaptive evolution, and how well species might adapt to future environmental change.

In the present study, we used two species in the genus *Begonia* to look for genomic patterns of adaptive divergence. *Begonia* is one of the most diverse genera of angiosperms, comprising more than 2000 species (Hughes *et al.*, 2015–) that occupy a wide range of ecological niches throughout the tropics and display diverse morphologies and vegetative forms (Burt-Utley, 1985). Studies of the origins of diversity in *Begonia* suggest a prominent role of genetic drift (Tseng *et al.*, 2019), the genus being characterised by typically poor seed dispersal mechanisms (Twyford *et al.*, 2014), leading to strong intraspecific population structure and high incidence of endemism (Matolweni *et al.*, 2000; Hughes *et al.*, 2003; Hughes & Hollingsworth, 2008; Nakamura *et al.*, 2012). Nevertheless, adaptive traits such as secondary woodiness (Kidner *et al.*, 2016), deceit pollination (Castillo *et al.*, 2012) and tolerance to high altitude (Barrera *et al.*, 2019) are evidence of at least a partial role for selective forces in shaping this megadiverse genus.

The two species of *Begonia* used in our study present an excellent model for finding signatures of divergent adaptive traits, due to their morphological and ecological differences yet close phylogenetic relationship. *Begonia conchifolia* A.Dietr. (**Figure 1**A) is a small terrestrial plant with long-lived fleshy peltate leaves and small white flowers. It has a restricted distribution in wet rain forests across southern Mexico and Central America, sometimes establishing itself successfully on open roadbanks. It often grows epiphytically on the lower parts of tree trunks or is saxicolous on rock faces uncolonised by other vascular plants. *Begonia plebeja* Liebm. (**Figure 1**B), which also has a terrestrial growth form, is more widespread, occupying seasonally dry, deciduous forests in northern Mexico, tolerating higher levels of insolation than typical for the genus. It has larger, thinner leaves,

Figure 1. The study species: A, *Begonia conchifolia*; *B. plebeja*. Photographs: Catherine Kidner.

which are deciduous in some populations and often blotched, and larger flowers sometimes tinged with pink (Burt-Utley, 1985).

In the present study, we used a comparative transcriptomics approach to investigate whether the ecological divergence between *Begonia conchifolia* and *B. plebeja* is accompanied by adaptive divergence in environmentally relevant functional gene categories. Using multitissue transcriptome sequencing data from *Begonia conchifolia* and *B. plebeja* (Emelianova *et al.*, 2021), we aimed to address whether there are species-specific signatures of gene expression, and whether such signatures could explain the difference in the niches occupied by the two species.

Materials and methods

Transcriptome sequencing and annotation

Reference transcriptomes for *Begonia conchifolia* (RBGE accession no. 20042082) and *B. plebeja* (RBGE accession no. 20051406) were assembled and annotated as described in a previous study (Emelianova *et al.*, 2021). Briefly, six tissues were chosen for sequencing: female flower, leaf, male flower, petiole, root and vegetative bud. RNA was extracted using a phenol chloroform protocol (Logemann *et al.*, 1987), using three biological replicates per tissue and species. TruSeq mRNA-seq libraries were prepared and sequenced by Edinburgh Genomics (Edinburgh, UK) on one lane of an Illumina HiSeq Rapid v1 machine (Illumina, San Diego, California, USA), generating c.240 million 150–base pair paired-end total reads. Adapter-trimmed reads were subject to contaminant screening using BlobTools (Laetsch & Blaxter, 2017), retaining only reads that mapped to Streptophyta for all downstream analyses.

Total reads were assembled using Trinity v2.6.4 (Grabherr *et al.*, 2011) to produce reference transcriptomes for *Begonia conchifolia* and *B. plebeja* (**Table 1**). The longest transcripts per locus (unigenes) were annotated using the Trinotate pipeline (Bryant *et al.*, 2017).

Table 1. Assembly statistics for *Begonia conchifolia* and *B. plebeja* transcriptomes

Variable	*B. conchifolia*	*B. plebeja*
Total no. of assembled sequences[a]	42,614	59,106
No. of assembled unigenes[b]	17,012	19,969
Shortest assembled sequence (bp)	201	201
Longest assembled sequence (bp)	15,923	16,037
N > 1 kb	31,876	37,865
N > 10 kb	28	16
N with open reading frame	32,848	43,119
N90	1090	842
N50	2381	1905

[a] All isoforms of reconstructed transcripts.
[b] Unigene defined as the longest isoform from each unique transcribed locus.

Read mapping

Reads from each tissue replicate in *Begonia conchifolia* and *B. plebeja* were mapped back to their corresponding species' reference transcriptome unigenes, using STAR v2.5.3a (Dobin *et al.*, 2013) with default parameters. Reads were counted using Subread's FeatureCounts v1.5.2 (Liao *et al.*, 2014), excluding read pairs that mapped to different unigenes.

Differential expression

For each of the two species, EdgeR (Robinson *et al.*, 2010) was used to identify differentially expressed genes (DE genes; i.e. genes differentially expressed between two types of tissue). Raw read counts generated by FeatureCounts were first filtered, retaining only unigenes that had more than five counts per million mapping to at least two samples. Samples were normalised for library size and composition, using TMM normalisation. The exact test was used to identify DE genes for each tissue-pair comparison, based on a significance level of adjusted *p* value (false discovery rate, FDR) < 0.05 and log2 fold change > 2.

Gene ontology enrichment analysis

Data on DE genes were subjected to gene ontology (GO) enrichment analysis with GOseq (Young *et al.*, 2010), which accounts for sequence length bias, using all unigenes in the corresponding species' reference transcriptome as a background. A corrected *p* value (FDR) threshold of 0.05 was applied when selecting significantly enriched GO terms.

To compare expression profiles between *Begonia conchifolia* and *B. plebeja* in the absence of a reference genome for both species, we used GO-term analysis to provide a broad overview of the types of DE genes. To identify GO terms shared by both species or unique to either species, GO terms were mapped for all DE genes identified for all tissue-pair comparisons.

We explored qualitative differences between *Begonia conchifolia* and *B. plebeja* in terms of DE genes. First, we used GOseq to identify GO terms enriched in the DE genes identified for each tissue-pair comparison for each species. Second, for each tissue-pair comparison (e.g. root versus vegetative bud), we sought to identify GO terms enriched in one species but not the other (these are hereafter referred to as uniquely enriched terms, UETs).

Annotation of DE genes underlying UETs related to light response was carried out using transcriptome annotations generated in a previous study (Emelianova *et al.*, 2021).

Results

Differential expression

For each tissue-pair comparison, the number of genes differentially expressed between the tissues was found to be broadly similar in *Begonia conchifolia* and *B. plebeja*, with both species sharing tissue-specific patterns (**Figure 2**). Data for both species showed differential

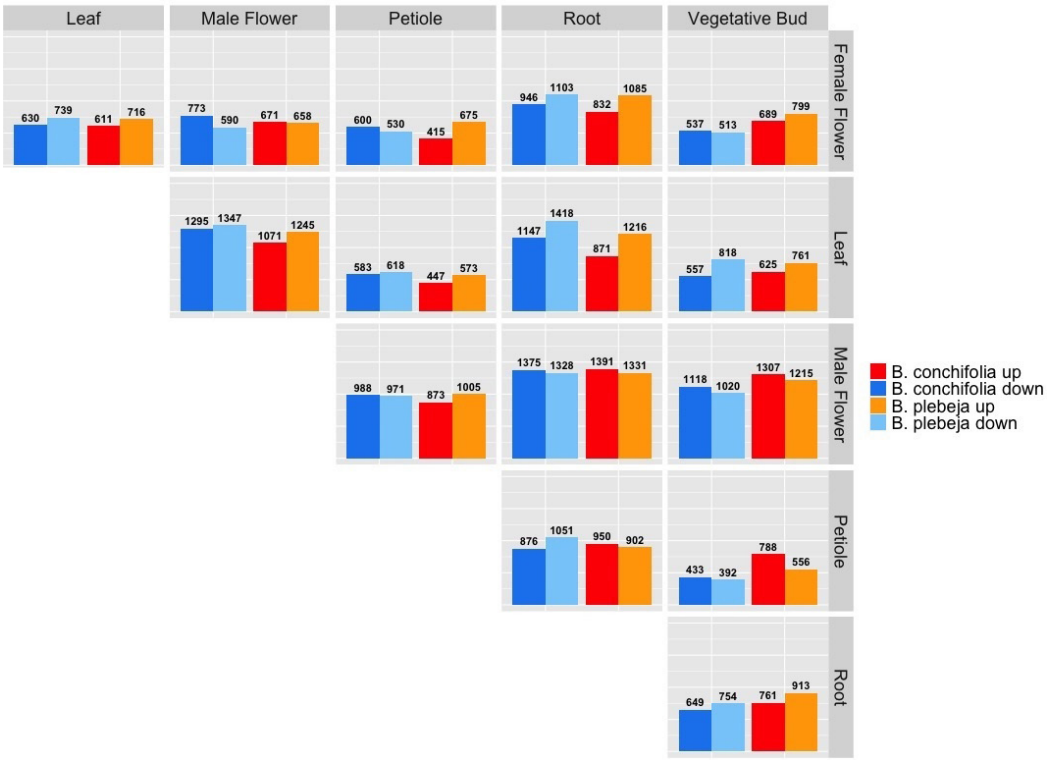

Figure 2. Barplot grid showing data for genes differentially expressed between different types of tissue in *Begonia conchifolia* and *B. plebeja*. Above each column is the number of differentially expressed genes for that tissue-pair comparison. Rows denote the base tissue and columns denote the comparison tissue. Up-regulated genes (red for *Begonia conchifolia* and orange for *B. plebeja*) and down-regulated genes (dark blue for *B. conchifolia* and light blue for *B. plebeja*) represent a significant increase or decrease, respectively, in expression in the comparison tissue relative to the base tissue. A significance level of adjusted *p* value (false discovery rate) < 0.05 and log2 fold change > 2 was used to define differential expression of a gene.

expression to be greatest between reproductive tissues (male flower or female flower) and roots; fewer genes were found to be differentially expressed between reproductive tissues and other vegetative tissues (petiole, vegetative bud and leaf).

Gene ontology enrichment analysis

The results of mapping of GO terms for all DE genes identified for all tissue-pair comparisons showed that of the total 5267 GO terms, 3694 mapped to DE genes in both *Begonia conchifolia* and *B. plebeja*, 776 mapped to DE genes in *B. conchifolia* only, and 797 mapped to DE genes in *B. plebeja* only. As expected, the number of GO terms mapped to DE

genes per tissue-pair comparison (Figure 3) was proportional to the number of DE genes identified for that comparison (see Figure 2).

The results for most tissue-pair comparisons, including the directions of change in expression, were similar between *Begonia conchifolia* and *B. plebeja* (see Figure 3). However, for some comparisons there was a notable difference between the two species; for example, 48 genes are up-regulated in petiole compared with female flower in *Begonia plebeja*, versus 18 in *B. conchifolia* (see Figure 3).

For almost every tissue-pair comparison, the number of DE genes underlying UETs was found to be greater in *Begonia conchifolia* than in *B. plebeja*, and these genes were shown to differ proportionally between comparisons in *B. conchifolia* versus *B. plebeja* (Figure 4). Genes underlying UETs in *Begonia conchifolia* were shown to predominate in comparisons

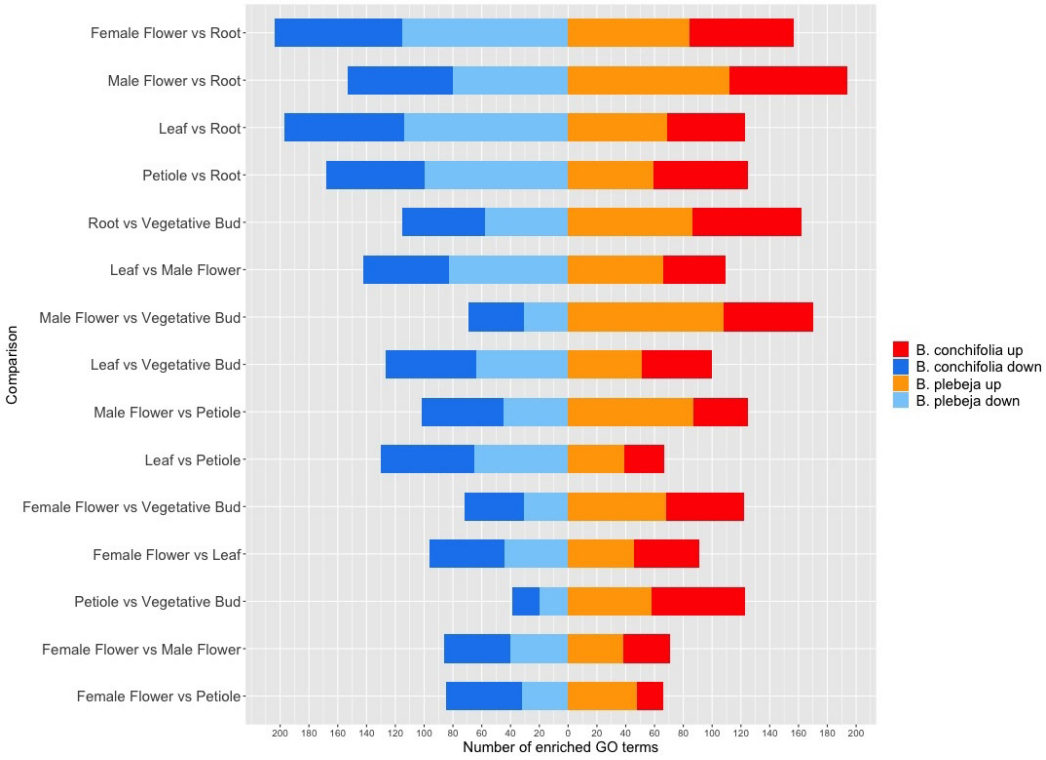

Figure 3. Number of gene ontology (GO) terms enriched in differentially expressed genes in *Begonia conchifolia* and *B. plebeja*, by tissue-pair comparison. The first and second tissue in each comparison are the base and comparison tissue, respectively. The number of GO terms mapped to up-regulated genes indicates the number of genes with signficantly greater expression in the reference tissue than in the base tissue. Conversely, the number of GO terms mapped to down-regulated genes indicates the number of genes with significantly lower expression in the reference tissue than in the base tissue.

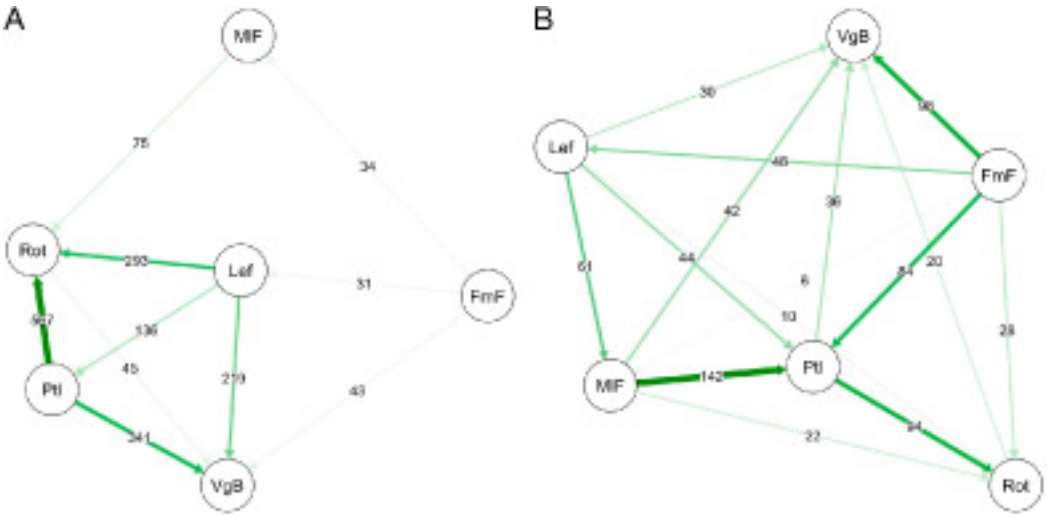

Figure 4. Network diagrams showing the number of genes associated with uniquely enriched gene ontology (GO) terms for each tissue-pair comparison carried out for *Begonia conchifolia* (A) and *B. plebeja* (B). For each comparison, the arrow indicates the direction of comparison, and the number is the number of genes underlying uniquely enriched terms. FmF, female flower; Lef, leaf; MlF, male flower; Ptl, petiole; Rot, root; VgB, vegetative bud.

between vegetative bud, petiole and root, whereas in *B. plebeja*, genes underlying UETs were more evenly distributed among the tissue-pair comparisons. Uniquely enriched terms were identified in fewer tissue-pair comparisons in *Begonia conchifolia* than in *B. plebeja* (10 in *B. conchifolia* versus 15 in *B. plebeja*).

In *Begonia plebeja*, compared with *B. conchifolia*, there was a greater propensity for UETs to be associated with a single tissue-pair comparison (Figures 5 and 6); analysis of the underlying data showed that 84% of *B. plebeja* UETs were associated with a single tissue-pair comparison, compared with 65% of *B. conchifolia* UETs. In both species, UETs associated with multiple tissue-pair comparisons were often found to have a tissue in common (see Figures 5 and 6). In the case of *Begonia conchifolia*, most shared terms were shown to be between petiole and vegetative bud, and between petiole and root (see Figure 5).

Our results showed that UETs in *Begonia conchifolia* tend to be related to housekeeping functions such as cell division, DNA replication and microtubule activity (**Figure 7**). Response to abscisic acid and response to water deprivation were among the few functional categories related to environmental responses (see **Figure 7**). Housekeeping functions such as cell division and photosynthesis were also represented in the *Begonia plebeja* UETs; however, a large proportion of these are related to environmental response, such as the ethylene-activated signalling pathway, comprising categories such as response to blue

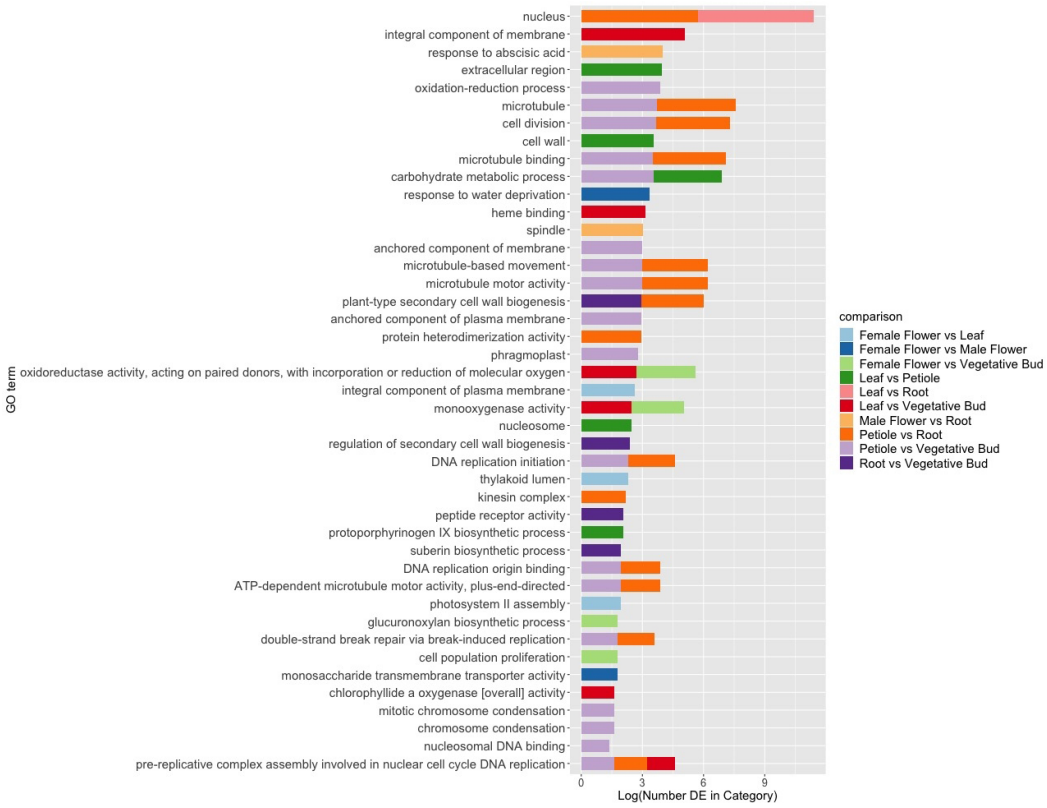

Figure 5. Numbers of genes associated with gene ontology (GO) terms that are enriched in differentially expressed genes (DE) for *Begonia conchifolia* but not *B. plebeja*, for each tissue-pair comparison. Gene numbers have been log transformed to improve the visibility of smaller GO categories. Up-regulated and down-regulated genes are included together within each group.

light and auxin influx (**Figure 8**). Other environmental response functions in *Begonia plebeja* include response to chitin.

Differentially expressed genes underlying UETs related to light response were further annotated for *Begonia plebeja*, as summarised in **Table 2**.

Discussion

Ecological heterogeneity provides a mosaic of different environmental niches, giving populations opportunities to undergo divergent adaptive evolution to take advantage of new habitats (Seehausen *et al.*, 2008). Regulation of gene expression is an important target of selection during adaptive evolution (Parry *et al.*, 2005; Jones *et al.*, 2012; Thompson *et al.*, 2018), and identification of the functional classes of genes involved in expressional

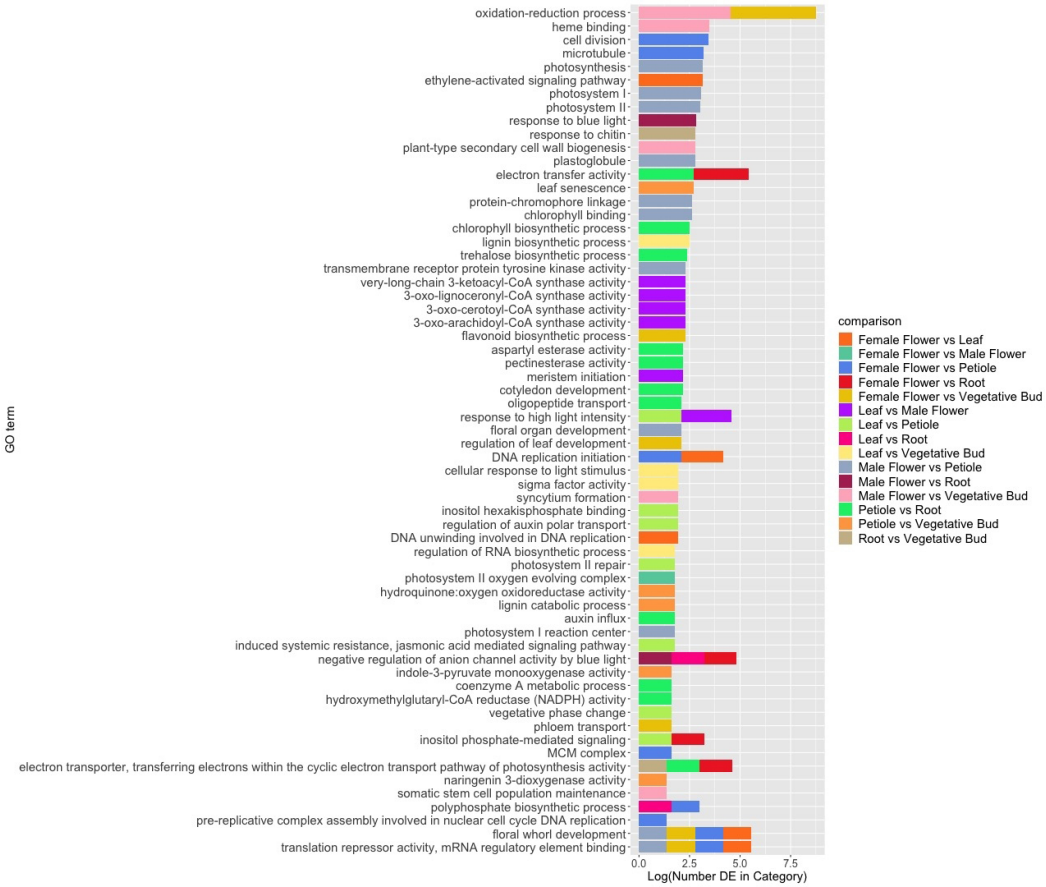

Figure 6. Numbers of genes associated with gene ontology (GO) terms that are enriched in differentially expressed genes (DE) for *Begonia plebeja* but not *B. conchifolia*, for each tissue-pair comparison. Gene numbers have been log transformed to improve visibility of smaller GO categories. Up-regulated and down-regulated genes are included together within each group.

divergence can reveal clues to the biotic or abiotic driving forces underlying divergent evolution (Dunning *et al.*, 2016; Piron-Prunier *et al.*, 2021).

Begonia conchifolia and *B. plebeja* represent an excellent model for studying the contribution of gene expression to ecological divergence and speciation; the morphological and ecological differences between the species are complemented by their relatively recent date of divergence, making it more likely that signatures of expressional divergence are detectable and not obscured by changes accumulated over longer time periods. Using our comparative transcriptome approach, we have identified key differences between *Begonia conchifolia* and *B. plebeja* in the expression of environmental response genes. Additionally,

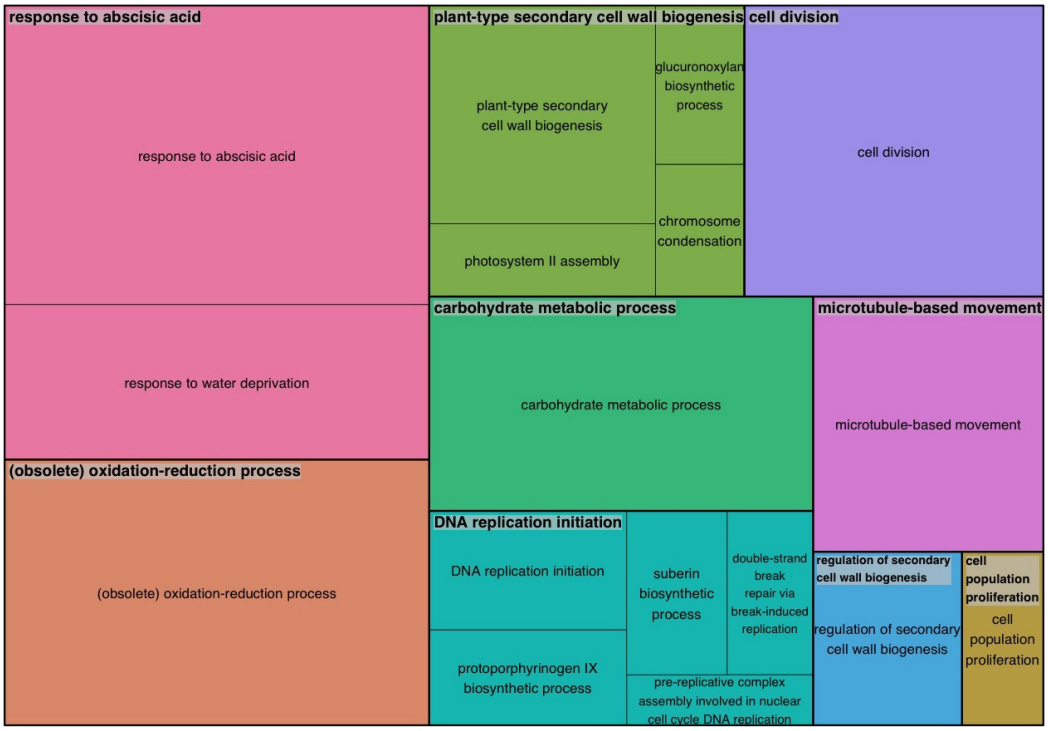

Figure 7. Gene ontology (GO) terms that are enriched in differentially expressed (DE) genes for *Begonia conchifolia* but not *B. plebeja* across all tissue-pair comparisons. The plot was generated using Revigo (Supek *et al.*, 2011), which visually summarises GO terms, removing redundant terms. Each colour-coded group of rectangles represents a cluster of loosely related terms; the size of each rectangle represents the number of significantly DE genes associated with that term.

we have identified species-specific expression of key light-regulated genes in *Begonia plebeja*.

A large proportion of light-related GO terms enriched in *Begonia plebeja* are involved in photomorphogenesis, the regulation of plant development in response to light. A substantial number of light-related genes annotated with GO terms enriched in *Begonia plebeja* are involved in the shade avoidance response, suggesting that this pathway is more active in *B. plebeja* than in *B. conchifolia*; thus, *B. plebeja* seems to have a more vigorous response to changing light availability. The shade avoidance strategy enables plants to cope with competition from neighbouring plants for light availability; foliar shade experienced during competitive over-topping is perceived by a reduction in the ratio of red to far red light wavelengths, triggering a regulatory cascade to prevent competitive light exclusion (Casal, 2013).

Phototropin-1 and phototropin-2 were among the *Begonia plebeja* genes annotated with light-related UETs. Members of the phototropin family of receptors are responsible for

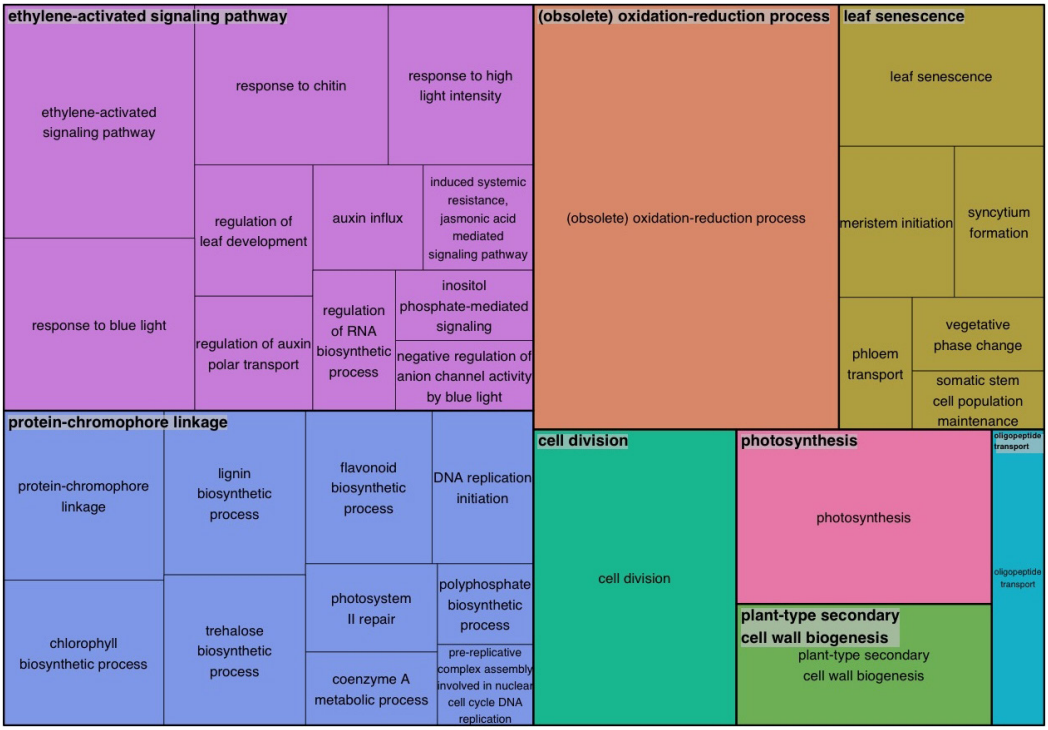

Figure 8. Gene ontology (GO) terms that are enriched in differentially expressed (DE) genes for *Begonia plebeja* but not *B. conchifolia* across all comparisons. The plot was generated using Revigo (Supek *et al.*, 2011), which visually summarises GO terms, removing redundant terms. Each colour-coded group of rectangles represents a cluster of loosely related terms; the size of each rectangle represents the number of significantly DE genes associated with that term.

sensing blue and red light and coordinating responses to optimise photosynthesis, including relocation of chloroplasts to enable their accumulation under low light (Sakai *et al.*, 2001) and movement to the cell walls under high light (Kagawa *et al.*, 2001). Responses to foliar shade include changes in reproductive strategy, via induction of early flowering (Galvão *et al.*, 2019), and redirection of resources to leaf and petiole elongation to thereby escape encroaching competitor foliar shade (Pierik *et al.*, 2009; Buti *et al.*, 2020b; Barillot *et al.*, 2021).

In genes annotated by *Begonia plebeja* UETs, we identified key genes in the basic/helix–loop–helix (bHLH) regulatory network, including the transcription factor bHLH63, which is involved in triggering early flowering in response to blue light (Buti *et al.*, 2020a), and HBI1, which positively regulates petiole elongation and early flowering (Zhiponova *et al.*, 2014). In the *Begonia plebeja* UET annotated genes, we also identified allene oxide cyclase, which catalyses the production of jasmonic acid, a key compound in the orchestration of photomorphogenesis in the jasmonate pathway (Nozue *et al.*, 2015; Yi *et al.*, 2020).

Table 2. Differentially expressed genes associated with gene ontology terms identified for *Begonia plebeja* uniquely enriched terms related to light response[a]

Response	Differentially expressed gene
Response to blue light GO:0009637	Cryptochrome-1
	Phototropin-2
	Phototropin-1
	Protein GIGANTEA
	ABC transporter B family member 1
	Transcription factor UNE10
	Protein LIGHT-DEPENDENT SHORT HYPOCOTYLS 1
	Transcription factor HBI1
	Transcription factor TCP2
	Homeobox-leucine zipper protein HAT5
	Phototropin-1B
	Protein SPA1-RELATED 4
	Allene oxide cyclase, chloroplastic
	Adagio protein 1
	Transcription factor bHLH63
	Allene oxide cyclase, chloroplastic
Cellular response to light stimulus GO:0071482	1,4-alpha-glucan-branching enzyme 1, chloroplastic/amyloplastic
	RNA polymerase sigma factor sigB
	RNA polymerase sigma factor sigF
	RNA polymerase sigma factor sigC
	RNA polymerase sigma factor sigA
Response to high light intensity GO:0009644	Calmodulin-binding receptor kinase CaMRLK
	Chlorophyll a-b binding protein 3
	Protein ACTIVITY OF BC1 COMPLEX KINASE 8
	Lysine-tRNA ligase, chloroplastic/mitochondrial
	Protein EARLY-RESPONSIVE TO DEHYDRATION 7
	Chlorophyll a-b binding protein 36
	Protein PROTON GRADIENT REGULATION 5
	Chlorophyll a-b binding protein 4
	Chloroplastic lipocalin
	Chlorophyll a-b binding protein 7
	Protein MET1
	Ferredoxin C 1
	Protein ACTIVITY OF BC1 COMPLEX KINASE 8
Negative regulation of anion channel activity by blue light GO:0010362	Phototropin-2
	Phototropin-1
	Phototropin-1B

[a] Annotations were obtained from *Begonia plebeja* annotations generated in a previous study (Emelianova *et al.*, 2021).

Begonia plebeja UET annotated genes also included key circadian rhythm–related genes, including blue light–dependent protein Adagio 1 (Somers *et al.*, 2000) and chaperone protein GIGANTEA (Dalchau *et al.*, 2011). Circadian clock genes fulfil an important negative feedback mechanism to prevent excessive light foraging, especially in shorter photoperiods (Franklin, 2020); identification of these transcripts in our dataset, along with other photomorphogenic repressors such as SPA1-RELATED 4 protein, provides greater insight into the coordinated regulation of shade avoidance in *Begonia plebeja*.

Our finding of UETS in fewer tissue-pair comparisons in *Begonia conchifolia* than in *B. plebeja* suggests less species-specific activity in the former. In both species, we found that UETs associated with multiple tissue-pair comparisons often have a tissue in common, suggesting that these functional terms may underlie a process specific to that tissue. In the case of *Begonia conchifolia*, we found most shared terms to be between petiole and vegetative bud, and petiole and root, and all related to cell division and housekeeping, which suggests that in this species, petiole is particularly divergent from other tissues in terms of rate of cell division.

Shade-tolerant species living in understorey habitats, such as *Begonia conchifolia*, have lower growth rates and reduced or absent elongation responses to shade (Morgan & Smith, 1979; Gommers *et al.*, 2013), and the UETs in *B. plebeja* suggest a higher turnover of plant growth and development (meristem initiation, leaf senescence, vegetative phase change) compared with in *B. conchifolia*.

Light availability is one of the most immediate differences in the habitats of our study species; *Begonia conchifolia* occupies a shaded understorey environment, with a steady supply of low diffuse light interspersed with periodic high-intensity sunflecks. By contrast, *Begonia plebeja* occupies a more open habitat in seasonally dry forests, with more access to direct, high-intensity sunlight (Burt-Utley, 1985). Environmental constraints of living under a canopy preclude a shade avoidance response, and therefore compel understorey dwelling species to optimise responses to a low-light environment (Valladares & Niinemets, 2008; Valladares *et al.*, 2016). Shade-tolerant species, being less sensitive to the ratio of red to far red light, are able to cope with low light levels without invoking a strong shade avoidance response (Kwesiga & Grace, 1986). Although less is known about shade tolerance than shade avoidance (Gommers *et al.*, 2013), some evidence exists for mechanisms to modulate shade avoidance responses. Preference of different phytochromes sensitive to far red, rather than red and blue light (Johnson *et al.*, 1994), as well as modulation of hormone pathways (Hornitschek *et al.*, 2012) and recruitment of shade avoidance antagonists (Buti *et al.*, 2020b), are among the various methods suggested to contribute to shade tolerance.

Using the approach described in this article, we have identified species-specific expression patterns in *Begonia conchifolia* and *B. plebeja*, revealing an active response

to shade avoidance in *B. plebeja*, and showing that, by contrast, *B. conchifolia* has little species-specific activity outside predominantly housekeeping functions. Thus, our work has uncovered a key difference in the responses of these two species to fluctuating light availability, and has laid the ground for further investigations to determine the role of light availability in the ecological divergence of *Begonia conchifolia* and *B. plebeja*.

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