Genetic Control of Heterochrony in *Eucalyptus globulus*

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ABSTRACT A change in the timing or rate of developmental events throughout ontogeny is referred to as heterochrony, and it is a major evolutionary process in plants and animals. We investigated the genetic basis for natural variation in the timing of vegetative phase change in the tree *Eucalyptus globulus*, which undergoes a dramatic change in vegetative morphology during the juvenile-to-adult transition. Quantitative trait loci analysis in an outcross F_2 family derived from crosses between individuals from a coastal population of *E. globulus* with precocious vegetative phase change and individuals from populations in which vegetative phase change occurs several years later implicated the microRNA *EglMIR156.5* as a potential contributor to this heterochronic difference. Additional evidence for the involvement of *EglMIR156.5* was provided by its differential expression in trees with early and late phase change. Our findings suggest that changes in the expression of miR156 underlie natural variation in vegetative phase change in *E. globulus*, and may also explain interspecific differences in the timing of this developmental transition.

KEYWORDS
QTL
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heterochrony
adaptation

Transition to the reproductive state is a key event in animal and plant development, and it is often accompanied by changes in somatic or vegetative traits. These changes may be rapid, synchronized, and dramatic, as seen in the well-known examples of animal metamorphosis and plant heteroblasty, or they may be more subtle, gradual, and unsynchronized. The timing of these transitions is usually under strong genetic control (Gould 1977; Guerrant 1988; MacKinney and MacNamara 1991) and can be subject to evolutionary change in a phenomenon referred to as heterochrony (MacKinney and MacNamara 1991). It has long been argued that markedly different morphologies in organisms of equivalent age may arise from relatively simple genetic changes in genes that control developmental timing, and that this may provide a means of rapid adaptive evolution (Gould 1977; Guerrant 1988; MacKinney and MacNamara 1991). Evidence for heterochronic evolution in plants in response to recent climate change is already emerging (Franks and Weis 2008).

Although heteroblasty is evident in many woody plant genera including *Hedera*, *Pinus*, and *Acacia* (Climent *et al.* 2006; Zotz *et al.* 2011), the evolutionary significance of heteroblasty is clearly exemplified in trees of the genus *Eucalyptus* (Barber 1965; Potts and Wiltshire 1997). Many *Eucalyptus* species are strongly heteroblastic, with a striking and abrupt change from the juvenile to the adult vegetative phase. Phase change in eucalypts is usually most obvious in leaf morphology and orientation but can also include changes in leaf anatomy, physiology, chemical composition, and resistance to pests and diseases, as well as changes in stem shape, bark type, and the anatomy and composition of wood (Potts and Wiltshire 1997; James and Bell 2001; Lawrence *et al.* 2003; Goodger *et al.* 2007; Jaya *et al.* 2010). This variation suggests that the different leaf phases in heteroblastic eucalypts may confer distinct advantages in specific biotic or abiotic environments (Jordan *et al.* 2000; James and Bell 2001). In eucalypts, the timing of vegetative phase change is under strong genetic control and may vary markedly both within and between species (Jordan *et al.*

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1999; Hamilton *et al.* 2011). Further, while reproduction is normally associated with the adult foliage type, at least 30 species from diverse eucalypt lineages are reproductive while bearing typical juvenile foliage in their canopy, and many of these never develop adult foliage (Potts and Wiltshire 1997). This process is likely to reflect heterochronic speciation. For example, it is thought that the rare Tasmanian endemic *E. risdonii* has recently speciated from the heteroblastic *E. tenuiramis* by retaining juvenile foliage for life (Wiltshire *et al.* 1998). Similar heterochronic differentiation separates other closely related species throughout the eucalypt phylogeny, suggesting this process has occurred independently numerous times in eucalypt evolution (Potts and Wiltshire 1997).

The forest tree *Eucalyptus globulus* is one of the best known heteroblastic plants and undergoes a dramatic vegetative phase change (Figure 1) (Hamilton *et al.* 2011; Zotz *et al.* 2011). *E. globulus* also provides a particularly striking example of heterochrony, with the occurrence of precocious reproductive and vegetative phase change in several dwarf coastal cliff-top populations (Figure 1) that are thought to have evolved in response to drought and salt stress and/or high winds (Dutkowski and Potts 1999; Jordan *et al.* 2000; Foster *et al.* 2007). Among these populations, the most extreme case of precocious reproductive and vegetative phase change occurs in the Lighthouse population at Wilsons Promontory in southeastern Victoria (Dutkowski and Potts 1999; Jordan *et al.* 2000). In common garden trials, most progeny from the cliff-top trees produce adult foliage and many have flower buds by the second year of growth, whereas progenies from most other populations, including adjacent disjunct populations, are taller and remain vegetatively and reproductively juvenile for 5 or more years (Jordan *et al.* 2000). However, despite much study of this ecotypic differentiation, the molecular basis of this variation is yet to be explored.

Although heteroblasty has been of interest to researchers for more than a century (Goebel 1889), it is only in the past 10 years that advances in molecular genetics have given insight into the underlying molecular mechanisms. Studies in *Arabidopsis* and maize have identified two miRNAs that act sequentially in a pathway that is central to regulation of vegetative phase change (Wu and Poethig 2006; Chuck *et al.* 2007; Wu *et al.* 2009). MiR156 promotes juvenility, is strongly expressed in seedlings, and its expression decreases during development, whereas miR172 is negatively regulated by miR156 and has the opposite role and expression pattern. MiR156 is nearly identical in sequence to miR157 and both target the same members of the *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*) gene family (Rhoades *et al.* 2002; Poethig 2009), while miR172 targets members of the *APETALA2* (*AP2*) family (Poethig 2009; Wu *et al.* 2009). Recent evidence suggests that the same miRNAs are involved in phase change in woody plants, including *E. globulus*, implying that they may regulate vegetative phase change in all flowering plants (Wang *et al.* 2011). However, although various studies have identified natural and induced mutants in which changes to these miRNAs or their targets affect phase change (Xie *et al.* 2005; Chuck *et al.* 2007; Usami *et al.* 2009; Wu *et al.* 2009), a link between polymorphism in genes involved in this regulatory network and adaptation in natural populations has not been demonstrated. Here, we aim to elucidate the molecular genetic basis of adaptive differentiation between *E. globulus* ecotypes with precocious vegetative phase change and those in which the timing of vegetative phase change is more characteristic of the species norm. We present evidence to suggest that genetic variation affecting the miRNA regulatory network plays a major role in ecotypic differentiation, highlighting the evolutionary importance of this regulatory network and heterochronic differentiation as an adaptive solution in plants.

MATERIALS AND METHODS

QTL study

Genetic material and trial design: Linkage mapping and quantitative trait loci (QTL) analyses were conducted in an inter-provenance outcross F_2 family (Lighthouse F_2). This family was constructed from crossing two unrelated F_1 individuals (the parents) in 2002, each derived from crossing individuals (the grandparents) with precocious [Wilsons Promontory Lighthouse (LH)] and "normal" [from either King Island in Bass Strait (KI) or Taranna in the southeast of Tasmania (TA)] vegetative phase change in 1995, thus producing the following pedigree for the F_2 family: 614LH/KI440//615LH/TA423. The F_2 family was planted at two sites, Boyer and Geeveston, approximately 50 km apart in southern Tasmania (Supporting Information, Table S1). Seed was sown in April 2006, with seedlings raised in glasshouse conditions before being planted in a randomized block design including various control families at Geeveston in November 2006, and at Boyer between May and July 2007. QTL analyses were conducted on a total of 467 F_2 individuals, 158 from Boyer and 309 from Geeveston.

Assessment and analysis of phenotypic traits: Vegetative phase change was scored in both trials at intervals of approximately 6 months, from trial planting until all trees had produced adult vegetation ($\sim$4.5 years of age). For the purpose of scoring, foliage was classified as juvenile, intermediate (bearing characteristics of both juvenile and adult foliage), or adult, as depicted in Figure S1. The timing of phase change was quantified by counting the number of nodes (cotyledons = node 0) at which transitions between these foliage types occurred, with the traits termed "last juvenile node" and "number of intermediate nodes." While measuring phase change traits, the height to each transition was also recorded to enable direct comparisons with previous studies (Jordan *et al.* 1999, 2000).

Flowering was quantified in terms of time and node that plants reached reproductive maturity, as well as the timing of flowering each year once reproductive maturity had been reached. These traits were scored in the flowering seasons beginning in 2009 and 2010. "Flowering precocity" represented a simple presence/absence of flowers at the end of the flowering year at each site and "node to first flower" was the node at which the first flower bud occurred. The proportions of flowers open, and yet to open, were recorded at least fortnightly during each flowering year. This was then converted to a peak flowering time ("anthesis time") following the method of Jones *et al.* (2011).

Tree height was measured in June 2010. Tree shape was calculated as tree height divided by the maximum canopy width. Tree shape was calculated using measurements made at 23 months of age in both field trials. Height measurements and internode counts were used to calculate mean internode lengths for juvenile and intermediate vegetative phases. The Pearson correlation coefficients were calculated between traits using PROC CORR of SAS.

Linkage mapping and QTL analysis: QTL analyses were performed using the linkage map described by Hudson *et al.* (2012). Briefly, all linkage maps were constructed using JoinMap 4.0 (Van Ooijen 2006). Individual parental maps were built using 503 F_2 individuals before constructing a sex-averaged consensus map. The consensus map contained 50 microsatellite (SSR) and 1010 DArT markers. To reduce computational demands, a subset of these markers (391) was used for QTL analyses. Specifically, most 3:1 segregating DArT markers were removed from the consensus map while retaining all SSR markers and

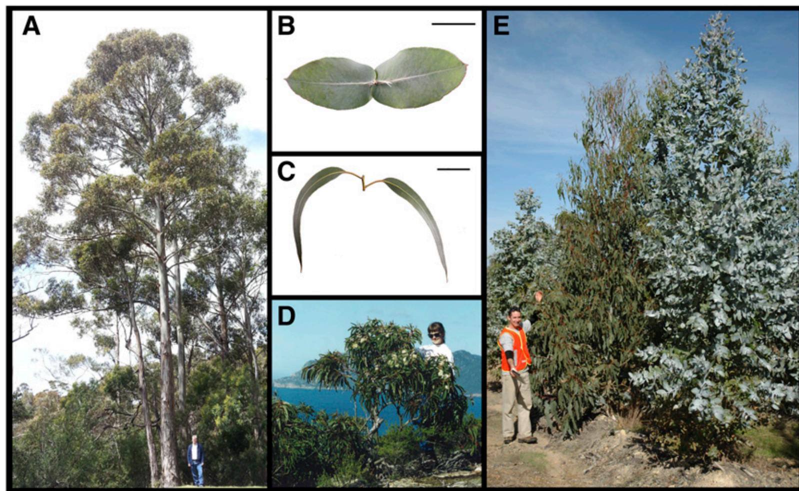


Figure 1 Heteroblastic and heterochronic variation in *Eucalyptus globulus*. *E. globulus* is usually a tall forest tree (A). However, on exposed granitic cliff-tops, *E. globulus* grows as a precocious ecotype that typically reaches less than 4 m and is often multi-stemmed (D). *E. globulus* undergoes a dramatic change in vegetative morphology from juvenile (B) to adult (C) foliage (scale bars indicate 5 cm). There is broad genetic-based variation in the timing of this transition (E) from precocious (left) to late (right) in ontogeny in *E. globulus*.

an even distribution of DArT markers segregating in a 1:1 ratio at approximately 2- to 5-cM intervals. Linkage group numbering corresponds to the 11 main scaffolds of the reference *E. grandis* genome sequence (v1.1 www.phytozome.net) (Myburg *et al.* 2014).

Putative eucalypt homologs of genes involved in vegetative and reproductive phase change in *Arabidopsis* were also mapped in this study (Wu and Poethig 2006; Wu *et al.* 2009; Chen *et al.* 2010; Wang *et al.* 2011). These included miR156, miR157, and miR172 loci, the genes targeted by these miRNAs, and genes involved in flowering (Table S2). As miR156, miR157, and miR172 precursor loci are not yet annotated in the *E. grandis* genome (Myburg *et al.* 2014), these were identified by performing a BLAST search with the mature miR156, miR157, and miR172 sequences to identify the stem loop sequence on Phytozome (http://www.phytozome.com/eucalyptus.php). Putative homologs of genes targeted by miR156/157 and miR172 were identified using the annotations on ORCAE (http://bioinformatics.psb.ugent.be/orcae/overview/Eugra) and Eucgenie (http://www.eucgenie.org/), respectively. Putative homologs of key flowering genes were identified by Vining *et al.* (unpublished data). All homologs were then placed on the linkage map based on extrapolation using the position of their closest flanking DArT markers in the *E. grandis* genome sequence (annotated at http://eucgenie.bi.up.ac.za/) using the "neighbors" approach (Cone *et al.* 2002). The average distance between each homolog genes and its flanking markers was 1845 kb.

QTL analyses were performed with MapQTL 6.0 (Van Ooijen 2009) using 467 individuals of the Lighthouse F_2. Phenotypic data were site-adjusted (site mean of zero; performed in SAS 9.2 using PROC STANDARD) and combined across sites for QTL analysis. Permutation tests were run in MapQTL 6.0 to determine LOD significance thresholds at genome-wide and chromosome-wide levels for all traits (1000 permutations) (Churchill and Doerge 1994). Putative QTL were declared at two significance thresholds: significant (genome-wide type I error $\alpha = 0.05$) and suggestive (chromosome-wide type I error rate $\alpha = 0.05$). Following the detection of putative QTL in interval mapping, markers linked to QTL that exceeded the suggestive ($\alpha = 0.05$) threshold were chosen as cofactors in restricted multiple-QTL model (rMQM) mapping. For each trait, rMQM analyses were performed using an iterative approach until no further QTL were detected, selected cofactor markers were the closest marker to each QTL, and QTL positions were stable. The regression algorithm implemented in MapQTL 6.0 was used in interval and rMQM mapping.

Population genomics analysis

An outlier marker analysis was conducted to detect potentially adaptive loci that differentiated the precocious Lighthouse population and the nearest normal population (Tidal River, approximately 14 km NW). The Lighthouse population is likely to have evolved from the adjacent Tidal River population (Foster *et al.* 2007) and was chosen to minimize differentiation due to stochastic processes, such as neutral drift, which generally increases with greater geographical/genetic separation. Thirty-two individuals from the Lighthouse population and 30 individuals from the Tidal River population were genotyped using DArTseq marker technology (DArT Pty. Ltd. Canberra, ACT) (Sansaloni *et al.* 2011). For each DArTseq marker, the average read depth was 9× and an absolute minimum sequence read depth of 3× in any individual/marker was used. BayeScan outlier analysis was conducted on 14,708 binary DArTseq markers following the removal of markers with the following: minor allele frequencies ≤2 out of 62 individuals (2875 markers) (Foll and Gaggiotti 2008; Roesti *et al.* 2012); more than 20% missing data in any one population; and quality scores (Q) <2. Outlier detection was performed using BayeScan v2.1 (Foll and Gaggiotti 2008; Foll *et al.* 2010; Fischer *et al.* 2011). Default BayeScan parameters were applied with a prior odds value of 10. Outlier markers were defined as those having a log10 (PO) value ≥0. Although this corresponds to a relatively weak level of outlier significance [a log10 (PO) ≥0<0.5 value is considered "barely worth mentioning" according to Jeffrey's Bayes factor interpretation], markers at this significance threshold were mapped to compare their position to detected QTL.

To estimate the position of outlier markers on the Lighthouse F_2 linkage map, BLAST searches were first performed (using the sequence supplied with each marker; all 69 bp in length) to identify the homologous position(s) of outlier markers in the *E. grandis* genome (http://bioinformatics.psb.ugent.be/webtools/bogas/). Linkage map positions were then estimated for each of these positions following the method of Cone *et al.* (2002).

Expression analysis

Ten plants of precocious phase change ecotype (from four open-pollinated families of the Lighthouse provenance) and nine plants of normal phase change ecotype (six plants from three open-pollinated families of Tidal River and three plants from a Taranna family related to a grandparent of the F_2) were grown in a randomized design in

a glasshouse under natural light. After 8 months, juvenile leaf material at node 10 of each plant (*i.e.*, 9–10 biological replicates) was harvested, frozen in liquid nitrogen, and stored at −70°. Plants were grown to monitor phase change phenotype; the normal ecotype remained juvenile after 2 years of growth (average 44 nodes), whereas all precocious ecotype plants had undergone phase transition by this stage.

Leaf material was crushed in liquid nitrogen in a mortar and pestle before nucleic acid extraction. RNA was isolated using the SV Total RNA Isolation System (Promega, Sydney, NSW, Australia) with the following modifications: 1% PEG (MW 20,000) was added to the lysis buffer, the optional 70° 3-min incubation step was included, and the DNase incubation was increased to 30 min. Genomic DNA was extracted using a CTAB method (McKinnon *et al.* 2004). Quality and quantity of nucleic acids were estimated using NanoDrop 8000 Spectrophotometer (Thermo Scientific). The Tetro cDNA Synthesis Kit (Bioline, London, UK) was used for cDNA synthesis, using 500 ng RNA in a volume of 20 μL. A negative control (omitting reverse-transcriptase enzyme) was included to confirm there was no genomic DNA contamination of RNA samples. Thirty microliters of RNase-free water was used to dilute cDNA for use in quantitative real-time PCR (qRT-PCR) or regular PCR. Primers for qRT-PCR (Table S3) were designed to target four miR156 precursor loci (*EgrMIR156.1*, *EgrMIR156.4*, *EgrMIR156.5*, *EgrMIR156.10*) identified by the BLAST searches of *E. grandis* described above using Primer3 v.0.4.0 (Rozen and Skaletsky 2000). Primers spanned the stem loop structure of each gene. Primers for *EglSPL3*, *EglSPL9*, and the housekeeping gene *EglEIF4* were those used by Wang *et al.* (2011) and primer sequences for another housekeeping gene, *EglACT2*, were provided by the same authors (M.Y. Park personal communication) (Table S3). Primers were first tested on *E. globulus* gDNA and cDNA using regular PCR (conditions as in Jones *et al.* 2011) using the annealing temperatures in Table S3. The resulting PCR products were cleaned and sequenced at the Australian Genome Research Facility (Brisbane, Queensland, Australia) to confirm the identity of the amplified product. Once confirmed in *E. globulus*, loci were renamed with the "Egl" prefix (Table S3). Sequences were deposited in GenBank, with accession numbers KJ948420 to KJ948423. Conditions used for qRT-PCR reactions were as in Jones *et al.* (2011). Each reaction was performed in duplicate and the C_q values of these two technical replicates were averaged. For the housekeeping genes *EglEIF4* and *EglACT2*, an unpaired two-tailed t test between mean C_q values for precocious and normal phase change phenotypes ($n = 10$ and $n = 9$ biological replicates, respectively; see description of genetic material above) was performed in GraphPad Prism Version 6.01 for Windows (GraphPad Software, La Jolla, CA), applying Welch's correction. These housekeeping genes did not differ in expression between the ecotypes ($P = 0.65$ for *EglEIF4* and $P = 0.79$ for *EglACT2*); therefore, *EglEIF4* was used for subsequent normalization of genes of interest. Reactions were normalized as in Hecht *et al.* (2011) and the t test between mean values for precocious and normal phase change phenotypes was conducted as described above.

Sequence analysis of *EglMIR156.5*

To investigate sequence variation in the *EglMIR156.5* region, a 1310-bp region encompassing the 87-bp *MIR156.5* stem loop and constituting part of the gene was sequenced in five Wilsons Promontory Lighthouse and five Tidal River individuals. Four primer pairs (Table S3) amplifying overlapping fragments were designed with PerlPrimer (Marshall 2004) and Primer3web (Koressaar and Remm 2007; Untergrasser *et al.* 2012). The template used for primer design was a consensus *E. globulus* sequence generated through the mapping of Illumina short-sequence reads (NCBI SRA library accession numbers SRX116786 and SRX059820; 54.8 G bases in total) to a 10-kb segment of the *E. grandis* BRASUZ1 genome sequence (version 1.1) that contained *EgrMIR156.5* (scaffold 3 from 50,817,705 to 50,827,704 bp). The short sequence reads were mapped using default settings in BWA-0.6.1 (Li and Durbin 2009) with the consensus sequence (average basepair read coverage of 23.5) being called with SAM tools (Li *et al.* 2009). PCR conditions were as in Jones *et al.* (2011) using the annealing temperatures in Table S3. PCR products were cleaned and sequenced at Macrogen (Seoul, South Korea) and aligned using Sequencher version 4.8 (Gene Codes Corporation, Ann Arbor, MI; http://www.genecodes.com). Sequences were deposited in GenBank, with accession numbers KJ933702 to KJ933711.

RESULTS

QTL analyses

To investigate the genetic basis of phenotypic differences between the precocious and normal ecotypes of *E. globulus*, we first conducted QTL analyses using a large outcross F_2 pedigree (n = 467) designed specifically to maximize segregation of ecotypic differences. In addition to traits related to vegetative phase change (last juvenile node and number of intermediate nodes), QTL analyses were also conducted for other traits that were likely to differentiate the ecotypes, namely: node to first flower; flowering precocity; anthesis time; tree height; tree shape; and juvenile internode length. In total, 28 QTL were identified exceeding the chromosome-wide significance level ($\alpha < 0.05$), of which two to five were detected for each trait (Table 1). QTL were located on all 11 linkage groups (Figure 2). In line with the strong correlations between many of the traits analyzed (Table S4), several QTL were co-located (*i.e.*, their 2-LOD support intervals overlapped) (Figure 2). Of the 28 QTL detected, nine were bi-parentally inherited, *i.e.*, segregated in both F_1 parents crossed to produce the F_2 (Table 1). We define these QTL as "ecotypic QTL" because their bi-parental inheritance indicated these were likely to be associated with population-level differentiation between the precocious and normal ecotypes. These nine ecotypic QTL were located on five chromosomes (with three co-located on LG3) and were associated with vegetative and reproductive phase change, juvenile internode length, and anthesis time.

Whereas most QTL were of relatively small effect [82% individually accounted for less than 5% of the phenotypic variation explained (PVE)], major effect QTL were detected for last juvenile node (62.8% PVE) and, to a lesser extent, for node to first flower (20.2% PVE). These QTL co-located with two other QTL for anthesis time and juvenile internode length on linkage group 3 (LG3) (Figure 2) and the results point to the effect of multiple loci in this region. The QTL for last juvenile node and node to first flower possibly represent the effects of a single pleiotropic locus because their peaks were close to each other (Table 1), their inheritance was bi-parental, their allelic effects were both additive (Figure 3), they segregated in coupling, and these traits were strongly positively correlated in the mapping family ($r = 0.64$; $P < 0.0001$). As would be expected, individuals inheriting alleles from both Lighthouse grandparents at these major QTL on LG3 underwent reproductive and vegetative phase change significantly earlier than heterozygotes or individuals homozygous for normal phase change alleles (Figure 3), indicating this locus strongly influences the timing of both transitions. However, the QTL for anthesis time and juvenile internode length were most likely controlled by different loci. This is supported by greater distances between peak positions for these QTL. Furthermore, the allelic effects of the QTL for juvenile internode length were dominant, and the QTL for anthesis time segregated from the female parent only, in contrast to the inheritance and mode of QTL effects at the co-located major effect QTL in this region (Table 1).

■ **Table 1 QTL detected in the Lighthouse *Eucalyptus globulus* F_2 family using rMQM mapping**

Trait	QTL					
	LG	cM[a]	Adj. marker[b]	LOD	PVE[c]	SEG[d]
Vegetative phase change traits						
Last juvenile node	2	32.4	ePt-641876	4.4**	1.6	F
	2	48.7	ePt-568767	4.7**	1.7	F
	3	62.1	ePt-639243	103**	62.8	Ecotypic
	5	48.6	ePt-641489	4.4**	1.6	M
	5	65.1	ePt-571521	4.0*	1.4	Ecotypic
Number of intermediate nodes	4	27.9	Es54	5.4**	3.8	Ecotypic
	6	34.8	Embra627	3.9*	2.5	F
	11	62.1	ePt-575083	9.1**	6.5	Ecotypic
Flowering traits						
Node to first	1	3.1	Embra11	3.3*	3.7	M
flower	3	61.0	ePt-639927	15.9**	20.2	Ecotypic
Flowering	4	66.9	ePt-568492	9.4**	5.2	F
precocity	7	65.3	ePt-504063	4.3*	2.4	F
	8	44.6	ePt-638446	5.4**	3.1	M
	8	78.7	ePt-640315	8.2**	4.6	Ecotypic
	10	70.9	ePt-572657	4.7**	2.6	M
Anthesis time	3	73.0	ePt-570139	4.7**	2.1	F
	4	72.4	Embra36	5.2**	2.4	Ecotypic
	5	0.9	Embra618	3.4*	1.5	M
	6	94.7	ePt-504481	5.2**	2.4	F
	8	129.5	Es76	8.0**	3.8	Ecotypic
Tree height and shape traits						
Tree height	3	42.4	ePt-571733	3.2*	2.7	F
	11	15.2	ePt-570063	3.6*	3.1	F
	11	39.5	Eg99	3.5*	3.0	F
Tree shape	4	54.2	ePt-564417	3.4*	3.0	F
	9	30.2	ePt-505052	4.4**	3.9	M
Juvenile	3	66.8	ePt-640855	15.6**	10.4	Ecotypic
internode	4	10.9	ePt-600106	3.8*	2.5	M
length	4	59.6	ePt-570676	5.8**	3.8	F

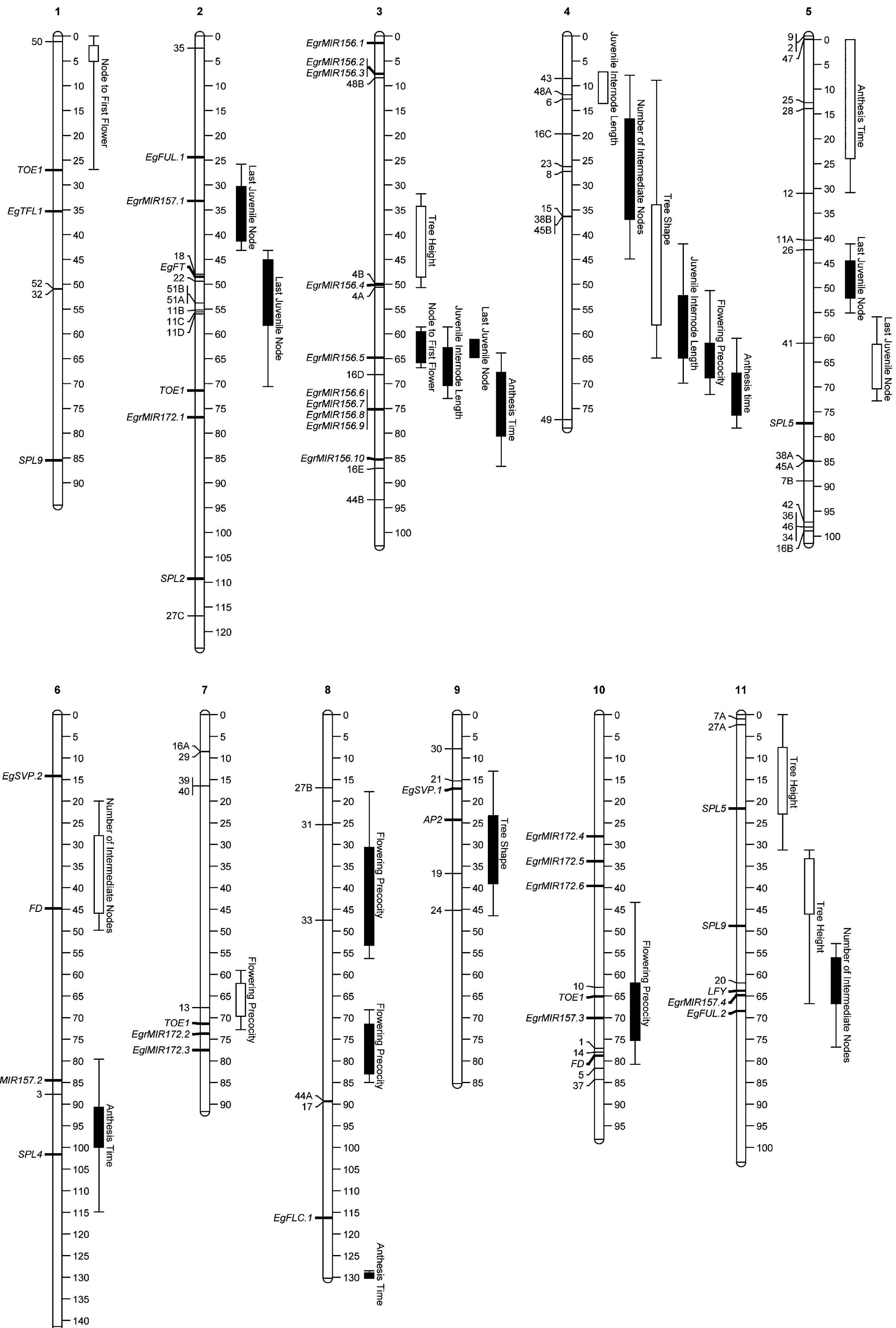
[a] QTL LOD peak position.
[b] Adjacent marker to QTL LOD peak.
[c] The percent variation explained for each QTL.
[d] Segregation of the QTL effect (M = male; F = female; Ecotypic = segregation from both parents or bi-parental). LOD significance: * = chromosome-wide $\alpha \leq 0.05$ and ** = genome-wide $\alpha \leq 0.05$.

Identification of candidate genes

In view of the central role of the miR156 pathway in regulation of vegetative phase change and flowering (Poethig 2009; Wu *et al.* 2009), we surveyed the entire *E. grandis* genome (v1.1 www.phytozome.net) for genes related to this pathway (miR156, miR157, and miR172 precursors, the target of these miRNAs, and flowering genes) as potential candidates underlying the major effect QTL on LG3. In *Arabidopsis*, there are eight miR156 precursors (*MIR156A–MIR156H*), four miR157 precursors (*MIR157A–MIR157D*), and five miR172 precursors (*MIR172A–MIR172E*) (Xie *et al.* 2005). These genes are not yet annotated in the *E. grandis* genome; our BLAST searches identified 10 miR156, four miR157, and six putative miR172 precursor loci in the main scaffolds of the *E. grandis* genome sequence (Table S2). The positions of these candidate genes were estimated on the Lighthouse F_2 linkage map (Figure 2) and these loci were distributed among 10 of the 11 linkage groups (Figure 2). Interestingly, all of the miR156 precursors (*EgrMIR156.1–EgrMIR156.10*) occurred on LG3, of which one (*EgrMIR156.5*) co-located with the major QTL for last juvenile node (Figure 2). Specifically, this gene was located within the region defined by markers spanning the 2-LOD confidence interval of the last juvenile node QTL, which corresponds to ~2.5 Mbp of the *E. grandis* genome sequence. We also searched this region of the *E. grandis* genome for other potential candidate genes. None of the annotated genes in this region (scaffold 3 from 48,300,000 to 50,840,000 bp, v1.1 www.phytozome.net, accessed February 18, 2014) had gene ontology related to, or an Arabidopsis homolog involved in, flowering or vegetative phase change (Table S5), providing evidence that the *EgrMIR156.5* locus is the best candidate gene in this region.

Expression and sequence analyses

Positional evidence from the QTL analysis implicated *EgrMIR156.5* as the potential cause of the difference in the timing of phase change between the precocious and the normal populations used in this study. To test this hypothesis, we used qRT-PCR to determine the abundance of the *EglMIR156.5* precursor in juvenile leaves of these ecotypes. The expression of *EglMIR156.5* was significantly (P = 0.0016) higher in juvenile leaves at node 10 of plants of normal phase change phenotype than in juvenile leaves at the same node on plants with the precocious phase change phenotype (Figure 4). This finding is consistent with the conserved role of miR156 suppressing vegetative phase change and provides further evidence that this locus underlies the observed difference in timing of vegetative phase change. In addition, transcript levels of the stem loop region of three other miR156 precursor loci (*EglMIR156.1*, *EglMIR156.4*, and *EglMIR156.10*) were



monitored in the same tissue. The expression of *EglMIR156.4* followed a similar trend to that of *EglMIR156.5*, but the difference between ecotypes was not significant (Figure 4). *EglMIR156.1* and *EglMIR156.10* were not or were poorly expressed in both ecotypes (Figure 4). To determine if the difference in expression of *EglMIR156.5* between ecotypes had an effect on genes downstream in the pathway, we analyzed the expression of the eucalypt homologs of *SPL3* and *SPL9*, genes that are inhibited by mature miR156 and promote vegetative phase change and flowering when mature miR156 levels are low (Chen *et al.* 2010). As expected, *EglSPL3* levels were higher in precocious compared with normal ecotypes, although this difference was not significant (Figure S2). *EglSPL9* was not expressed in either ecotype (Figure S2).

Sequence surrounding the *EglMIR156.5* stem loop (up to 1310 bp) was obtained for five Lighthouse and five Tidal River wild trees. No fixed polymorphic differences were detected between populations. The sequence(s) responsible for the differential expression of this gene may therefore reside outside the immediate stem-loop region.

Population genomics analysis

To further explore the genetic basis of ecotypic differentiation, a genome scan was performed using 14,708 DArTseq markers to identify "outlier markers" undergoing differential selection between the precocious Lighthouse population (n = 32) and the nearest normal population, Tidal River (n = 30). The position of outlier markers on the Lighthouse F_2 linkage map was estimated to enable comparison with the position of QTL and candidate genes, providing population-level positional support for some of the QTL identified as contributing to ecotypic differentiation. The mean population F_{ST} value between Lighthouse and Tidal River populations was 0.25, consistent with isolation and drift in the small Lighthouse population (Jones *et al.* 2013). In total, 59 outlier markers (0.4%) were identified at the log10 (PO) ≥ 0 significance threshold. Only markers with fixed, or near-fixed, differences in allele frequency between populations were detected as outliers. According to Jeffrey's Bayes factor interpretation, 16 outlier markers were significant at "strong" and "substantial" thresholds, respectively, with 27 markers significant at "the barely worth mentioning" threshold. For the 59 outlier markers, the maximum q-value at which all markers were significant was 0.23, meaning that 23% (or 14) of the outlier markers identified may be false positives.

At a minimum of 50 out of 69 bp (72%) sequence match, 52 of the 59 outlier markers returned BLAST matches to the 11 main scaffolds of the *E. grandis* genome. Ten markers returned multiple equal-quality sequence alignments [allowing for a maximum of two single nucleotide polymorphisms (SNPs) between alignments] (Table S6). Thus, the 52 outlier markers were mapped to 68 genomic positions, distributed across all 11 chromosomes (Figure 2). Importantly, outlier markers provided positional support for six of the nine ecotypic QTL (Figure 2). The co-location between ecotypic QTL and outlier markers occurred more commonly than expected by chance (χ^2_{df1} = 4.4; P = 0.035). These results provide independent validation of genomic regions identified in the QTL analysis, suggesting that many of the polymorphic loci with significant phenotypic effects in our F_2 family also differentiate the natural populations and are subject to selection. No significant outliers were located within the confidence intervals of the major-effect QTL for node to first flower and last juvenile node on LG3. However, a marker with nearly fixed differences in allele frequencies between the Lighthouse and Tidal River populations (fixed in Lighthouse and fixed except for two plants in Tidal River) occurred ~0.5 and 2.5 cM from the major-effect QTL peak for node to first flower and last juvenile node on LG3, respectively, supporting the identification of these QTL as key contributors to differentiation of the precocious and normal ecotypes.

DISCUSSION

Identifying the genetic basis of natural variation in developmental trajectories and understanding the evolutionary potential of this variation are major challenges of evolutionary biology. The demonstration that miRNAs regulate the timing of vegetative phase change not only in model annual plants but also across a wide variety of plant taxa including woody perennials (Huijser and Schmid 2011; Wang *et al.* 2011) now provides the possibility to explore the molecular basis of heterochronic evolution in natural populations. In this study we examined the molecular basis of heterochronic differentiation between naturally occurring ecotypes of the forest tree *E. globulus* with precocious vegetative phase change and those in which the timing of vegetative phase change is more characteristic of the species norm. Our results provide evidence suggesting that genetic variation affecting the miRNA regulatory network plays a major role in differentiating these ecotypes.

Polymorphism affecting the miRNA regulatory network underlies intraspecific variation in the timing of vegetative phase change in *E. globulus*

While functional genomic studies have provided detailed information regarding the genetic basis of a number of traits in model organisms, much less is known regarding how polymorphism at these loci contributes to adaptation in natural populations. Consistent with the role of miR156 as a "master regulator" of vegetative phase change in plants (Poethig 2010), our QTL and expression analyses provide independent evidence that genetic variation affecting this regulatory network underlies differentiation in the timing of vegetative phase change between the *E. globulus* ecotypes examined. Specifically, the detection of an ecotypic QTL for vegetative phase change (last juvenile node) of extremely large effect occurring adjacent to *EgrMIR156.5* implicates this locus as a positional candidate. The detection of a QTL for reproductive phase change at essentially the same genomic location (node to first flower) (Figure 2) provides strong additional support for this candidate locus, because the genes targeted by miR156/157 are some of the only loci known to influence both vegetative phase change and flower initiation (Huijser and Schmid 2011). Further support for the role of the miR156 regulatory network is

Figure 2 The location of QTL, candidate genes, and outlier markers on the Lighthouse F_2 linkage map in *Eucalyptus globulus*. Particularly notable is the co-location between the major QTL for last juvenile node and node to first flower on LG3 with *EgrMIR156.5*. Bars and lines indicate one-LOD and two-LOD QTL confidence intervals, respectively. QTL exceeding genome-wide ($\alpha \leq 0.05$) and chromosome-wide ($\alpha \leq 0.05$) significance thresholds are indicated by filled and empty bars, respectively. Significant outlier markers (Table S6) and candidate genes (Table S2) are shown on the left of linkage groups. Outlier markers are designated by a number followed by a letter (A–E) if they were present in multiple copies in the *E. grandis* genome sequence.

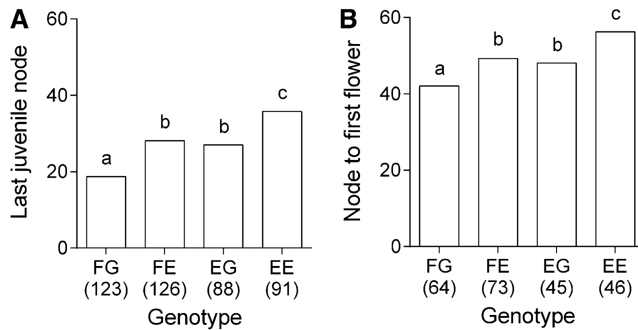


Figure 3 Co-dominant and bi-parental inheritance of the major QTL for vegetative and reproductive phase change in *Eucalyptus globulus*. Phenotypic means for last juvenile node and node to first flower are shown for each genotype class of Embra1656 (a microsatellite marker less than 3.4 cM from the QTL peak on LG3 for either trait). Genotype classes indicate the inheritance of alleles from grandparental ecotypes: FG = homozygous precocious (*i.e.*, contains two precocious grandparental alleles, inherited from Lighthouse 614LH and 615LH grandparents); FE = heterozygous genotype (615LH and KI440); EG = heterozygous genotype (614LH and TA423); and EE = homozygous normal (KI440 and TA423). The sample sizes of genotype classes are shown in parentheses. Letters above bars indicate significant differences (within traits; adjusted Tukey $\alpha = 0.05$) between genotype classes.

provided by the genetic-based difference in expression of *EglMIR156.5* between ecotypes with marked differences in the timing of vegetative phase change. In *Arabidopsis*, miR156 is highly expressed during early shoot development, suppressing vegetative phase change, and declines rapidly during the juvenile-to-adult vegetative transition (Yang *et al.* 2011). Therefore, our finding of significantly reduced expression of *EglMIR156.5* in juvenile leaves of *E. globulus* plants with precocious phase change, relative to those with normal phase change, is consistent with genetic variation affecting the miRNA regulatory network contributing to heterochronic differentiation.

Recent evidence from Arabidopsis suggests that only two of the eight miR156 precursor genes are likely to make a significant contribution to the overall level and developmental regulation of the mature miR156 (Yang *et al.* 2011, 2013; Yu *et al.* 2013). Similarly, of the four miR156 precursor loci we analyzed in *E. globulus*, only two, *EglMIR156.4* and *EglMIR156.5*, were expressed and only the latter varied significantly in expression between precocious and normal ecotypes. Because the relative contribution of individual miR156 precursor loci to the mature pool of miR156 and how this varies in different taxa are not well-understood, it is quite possible that in *E. globulus* ecotypic variation in the expression of *EglMIR156.5* alone could result in substantially reduced miRNA156 levels, and therefore precocious phase change.

Assuming that variation in the expression of *EglMIR156.5* underlies the major effect QTL differentiating precocious and normal phase change ecotypes, the lack of fixed sequence differences between these ecotypes directly in or around the stem-loop region suggests that the difference in expression of *EglMIR156.5* may be due to a mutation in parts of the gene we have not sequenced or in a *cis*-regulatory element near this locus. In other systems, causative mutations at loci with major phenotypic effects on developmental traits have been shown to lie outside the coding unit of the underlying gene, as in the case of the *Corngrass1* (Chuck *et al.* 2007) and *teosinte branched1* loci, which are both important in maize domestication (Clark *et al.* 2006; Studer *et al.* 2011). In the case of the severe *Corngrass1* mutant, which fixes plants in the juvenile stage, overexpression of the *miR156b/c* locus is caused by a retro-transposon insertion close to the transcription initiation site (Chuck *et al.* 2007). In the case of the transcription factor *teosinte branched1*, the causative mutations are also transposable element insertions but lie much further upstream, approximately 60–70 kb from the gene itself (Studer *et al.* 2011; Zhou *et al.* 2011).

Alternatively, it is formally possible that the QTL may represent the effect of a locus near but distinct from *EglMIR156.5*, which may act in *trans* to regulate expression of *EglMIR156.5* specifically or of multiple miR156 precursor loci. While our expression analysis of the miR156 precursor loci was not exhaustive, the fact that *EglMIR156.5* was the only locus to display differential expression between precocious and normal phase change ecotypes is consistent with the regulation of this locus alone. Another possibility is that a distinct mutation in the QTL region could affect *EglMIR156.5* expression through local alteration in chromatin structure. Clearly, without the identification of an obvious causal polymorphism, the evidence that the major effect QTL reflects a mutation in the *EglMIR156.5* gene or in a *cis*-regulatory element near this locus is inconclusive. Nonetheless, it remains the most plausible explanation from the available evidence. Future expression analysis of loci surrounding *EglMIR156.5* and additional miR156 precursor loci, as well as detailed sequence analysis of the wider genomic region surrounding *EglMIR156.5*, should shed more detail on the exact nature of the genetic differentiation between the ecotypes examined.

Evolutionary implications

Heterochrony has been widely implicated in the adaptive evolution of a diverse group of woody perennial genera (Li and Johnston 2000;

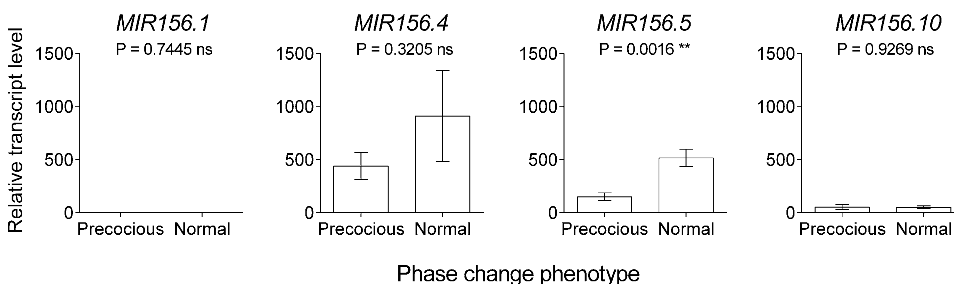


Figure 4 Expression of four miR156 precursors in precocious and normal phase change ecotypes of *Eucalyptus globulus*. The qRT-PCR analyses of miR156 precursors were performed using node 10 juvenile leaves. Expression was normalized as the percentage of expression above *EglEIF4* expression. Shown are the mean and SE of the expression in 10 plants of precocious phase change phenotype (representing four families from the Wilsons Promontory population) and nine plants of normal phase change phenotype (representing four families from Tidal River and Taranna provenances). *Significant difference between mean values of *EglMIR156.5* expression for precocious and normal phase change phenotypes in the *t* test; ** = $\alpha \leq 0.01$. Expression of *EglMIR156.1* was so low that it is not visible here.

Climent *et al.* 2006), including *Eucalyptus* (Barber 1965; Potts and Wiltshire 1997; Wiltshire *et al.* 1998), and numerous studies have argued that the timing of vegetative phase change, in particular, has adaptive significance in eucalypts (Wiltshire *et al.* 1991; Jordan *et al.* 2000; Hamilton *et al.* 2011). In *E. globulus*, it has been argued that the retention of juvenile foliage may be advantageous in mesic environments, whereas early transition to the more xeromorphic adult may confer an advantage in environments where water is limited (Jordan *et al.* 2000). The main selective forces in the cliff-top environment inhabited by the precocious ecotype are likely to be exposure to strong winds, salt spray, and drought (King 1999; James and Bell 2001). Plants can have similar physiological responses to drought and salt stress because both limit water uptake (Munns 2002), whereas wind exposure causes both drought and mechanical stress. Therefore, early phase change may have provided trees with a selective advantage by reducing susceptibility to desiccation and wind damage earlier in development. Outlier markers were located within three of the five QTL for last juvenile node, consistent with differential selection acting on the timing of phase change between the different ecotypes.

The outlier analysis identified many genomic regions under differential selection between the ecotypes. Many of these outliers were located within QTL confidence intervals, pointing to the possibility that these traits are also under differential selection between the ecotypes. Additionally, many outlier loci did not co-locate with QTL. Some of these outliers are likely to reflect variation in phenotypic traits that were not measured in this study, but are under differential selection between the ecotypes. Taken together, these results indicate that ecotypic divergence has involved various distinct traits and many genomic regions. Given that linkage disequilibrium (LD) probably decays rapidly in *E. globulus*, as in most tree species (Thavamanikumar *et al.* 2013), it is likely that many more markers than we used (14,708 markers) would have been necessary to saturate the genome for exhaustive outlier marker detection. Our low marker coverage probably explains why we did not find an outlier marker near *EglMIR156.5*. However, the low marker coverage also means that we have most likely underestimated the number of genomic regions involved in the differentiation of our ecotypes.

While it has long been argued that heterochronic evolution can produce marked changes in morphology through few genetic changes (Gould 1977; Guerrant 1988; MacKinney and MacNamara 1991), to date there has been little direct proof for this. Our findings address this long-standing issue. The major QTL for phase change had an exceptionally large effect (62.8% PVE) when it is considered that individual QTL generally account for less than 5% of variation in most quantitative traits in forest trees, crop plants, and humans (Visscher 2008; Buckler *et al.* 2009; Neale and Kremer 2011), as was the case for the majority of the QTL detected in this study. This implies that the underlying gene or genes is likely to have profound effects on the timing of vegetative phase change. The segregation of QTL effects from both F_1 parents indicates that alleles at this locus differentiated both grandparents with the precocious ecotype from those with normal phase change. Such loci with major effect have also been found to underlie variation in developmental traits in salamander [*Pisum sativum* (Wiltshire *et al.* 1994); *Ambystoma* sp. (Voss and Smith 2005); and *Zea mays* (Chuck *et al.* 2007)], collectively supporting the potential for rapid heterochronic evolution with few genetic changes.

While our results suggest ecotypic differentiation is a result of genome-wide adaptation, and most differences involve QTL of small effect (consistent with the bulk of plant and animal studies of ecotypic differentiation or early speciation reported to date), it is possible that the major effect QTL linked to the microRNA gene was the primary mutation that allowed the colonization and persistence of *E. globulus* in an ecologically extreme coastal habitat. Under this scenario, following the establishment of *E. globulus* with the precocious mutation in the cliff-top habitat (*e.g.*, Wilsons Promontory), limited gene flow with subsequent selection may have led to a gradual build-up of genome-wide adaptation to produce the contemporary pattern of genomic differentiation that distinguishes the precocious Wilsons Promontory population from the adjacent "normal" Tidal River population. By focusing on a natural system where variation in the timing of vegetative phase change appears to be an important adaptation, we highlight the potential evolutionary importance of the miR156 regulatory network in heterochronic evolution in flowering plants.

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