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Genetic mapping of *Eef1*, a major effect QTL for early flowering in *Eucalyptus grandis*

Abstract An early flowering mutant plant of *Eucalyptus grandis* with normal vegetative growth was found in a nursery in northern Brazil. This mutant plant flowers at approximately 90 days from germination. A cross between a wild-type (normal flowering) tree and the mutant was carried out, generating a progeny of 88 individuals where early flowering segregated in an approximate 1:1 ratio. A genome scan with 100 microsatellite markers distributed across the genome was carried out using bulk segregant analysis (BSA) on two contrasting bulks of 15 plants each. Linkages (LOD>3.0) with a major effect early flowering quantitative trait locus (QTL) were detected and confirmed by a full scale cosegregation analysis for markers EMBRA27, EMBRA60, EMBRA164, EMBRA158, EMBRA91, and EMBRA65. A localized linkage map involving the six loci and the early flowering QTL named

Eucalyptus early flowering 1 (*Eef1*) was constructed belonging to linkage group #2 in the existing microsatellite reference map. The *Eef1* locus was mapped between markers EMBRA27 and EMBRA164, with distances of 21.8 and 6.4 cM, respectively. In introgression experiments, these two markers could be successfully used with an expected precision of 98% to select plants carrying the *Eef1* mutant allele, assuming no recombination interference in the genomic segment. Early flowering could be a very useful trait both in breeding as well as experimental genetics of *Eucalyptus*.

Keywords *Eucalyptus grandis* · Early flowering mutant · Bulk segregant analysis

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Introduction

Early flowering has long been considered a desirable trait for shortening generation intervals in breeding programs of *Eucalyptus*. In *Eucalyptus grandis*, first flowering generally takes place at 2 to 3 years. Some species from cooler climates such as *Eucalyptus globulus*, *Eucalyptus nitens*, *Eucalyptus regnans*, and *Eucalyptus dunnii* do not flower heavily even at wide spacing until 7 to 10 years [12]. A significant development in recent years has been the use of paclobutrazol, a gibberellin inhibitor, to induce abundant and early flowering at less than 2 years of age without degrading seed quality in mature *E. globulus* and *E. nitens* [17, 18, 26]. This plant hormone has also been applied successfully to tropical *E. grandis* and *Eucalyptus urophylla*, although usable flowering still only occurs at age 12 to 18 months (T.F. de Assis, personal communication). Paclobutrazol, however, is resistant to chemical and biotic degradation [20] persisting in the soil for several years [17], making the long-term effects of this component on plant growth a concern.

In *Arabidopsis*, genetic and physiological studies related to reproductive onset have revealed the existence of more than 80 genes involved in the regulation flowering time, all of them responding to endogenous signals and environ-

mental changes [30]. It is now well established that inappropriate flowering in *Arabidopsis thaliana* is prevented mainly through the regulation of the floral inhibitor flowering locus C (FLC) that encodes a MADS domain transcription factor that directly represses a series of flowering-promoting genes [19]. Although FLC is a potential candidate gene involved in early flowering mutant phenotypes, to our knowledge, no FLC genes have been identified outside the *Brassicaceae* family.

Despite intensive research on the molecular genetic control of flowering time in model and some annual plants, only a few studies have been carried out on aspects of this process in forest trees. Most molecular studies involved the isolation of putative orthologs to known *Arabidopsis* flowering genes, followed by the analysis of their effectiveness to alter flowering phenotypes when overexpressed in transgenic plants. The *Arabidopsis* floral meristem identity gene, LEAFY (LFY) with a known ability to accelerate flowering when overexpressed [33], has been a target of considerable interest toward obtaining early flowering trees. A *Eucalyptus* LEAFY ortholog (ELF1) cloned from *E. globulus*, when overexpressed in transgenic *Arabidopsis*, caused early flowering, suggesting that ELF1 plays a similar role to LFY in flower development, and that the basic mechanisms involved in flower initiation and development in *Eucalyptus* are similar to those in *Arabidopsis* [31]. Furthermore, the expression of two *Eucalyptus* homologs of the *Arabidopsis* floral homeotic gene AP1 (EAP1 and EAP2) in *Arabidopsis* transgenics caused plants to flower earlier [24]. Recently, an *E. grandis* ortholog of LEAFY (EgLFY) was also cloned showing 95.2% identity to the previously published ELF1 gene [10]. In Poplar, although the ectopic expression of a *Populus trichocarpa* homolog of LEAFY (PTLFY) accelerated flowering in *Arabidopsis*, only one of the many tested transgenic lines of *Populus* flowered precociously, suggesting that PTLF activity appears to be subject to regulation that does not affect heterologously expressed LFY and is dependent upon tree maturation [28].

Little is known on the genetics of flowering in *Eucalyptus*. High levels of genetic differentiation were observed among natural populations of *E. globulus* for the timing of both transition to adult foliage [11] and first flowering [8]. Furthermore, in the *Eucalyptus tenuiramis* complex, for example, first flowering in a common environment experiment varied among families from 18 months to at least 8 years [35]. Analyses of large, open-pollinated, and controlled-cross field trials of *E. globulus* showed quantitative genetic independence of the times of first flowering and the abrupt change in leaf form, both these traits under moderate to strong additive genetic control [21].

The identification of natural mutants in forest trees is not an easy task due to their large size, long generation times, and delayed expression of target traits. A well-known case is a mutant loblolly pine *(Pinus taeda* L.) in which expression of the gene encoding cinnamyl alcohol dehydrogenase was severely reduced [24]. To our knowledge, no early flowering mutants have been yet reported for *Eucalyptus* or for any other forest plantation tree species.

Anecdotal reports are common from Brazilian nurseryman about early flowering seedling in *E. grandis*. Eldrige et al. [12] also mention observations of 2-month-old seedlings bearing flowers in tropical conditions pointing out that descendants of these plants were just as precocious, suggesting that early flowering is strongly inherited. In this work, we describe the genetic mapping of an early flowering major effect quantitative trait locus (QTL) named *Eucalyptus* early flowering 1 (*Eef1*) inherited as a dominant trait from a mutant *E. grandis* tree A0085. This QTL allele is inherited in a mendelian fashion and causes flowering to occur at approximately 60 to 90 days from germination.

Material and methods

Genetic material and phenotyping An early flowering *E. grandis* (Atherton provenance) seedling (A0085) was originally identified in the nurseries of Celmar S.A. Company, currently Ferro Gusa Carajás S.A. Company, in the municipality of Imperatriz, Maranhão State, latitude 47°26′30″ West 5°34′15″ South, in 1998. Due to its good growth, adaptability, and form in the context of a breeding program, the mutant was crossed to a wild-type normal flowering tree of *E. urophylla* (U0243). The early flowering phenotype, determined by the appearance of viable flower buds between 60 and 90 days from germination (Fig. 1), was inherited by the progeny in an approximate 1:1 ratio. The phenotype was scored in a binary fashion, presence or absence of flower buds, at 120 days from germination. Plants that did not flower by that age did not show flowering in subsequent days, although eventually they flowered at age 20 to 24 months, the regular flowering age in tropical conditions.

Microsatellite genotyping DNA extractions from expanded leaves were carried out as described previously [15]. Microsatellite genotyping was carried out in 13 μl reaction containing 1× PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 0.19 μM dNTP, 0.24 μg/μl bovine serum albumine (BSA) (New England Biolabs),

Fig. 1 Photographs of a progeny individual of the early flowering mutant *Eucalyptus grandis* (A0085) (Atherton provenance). *Left photo*, view of a 5-month-old seedling planted in the field displaying abundant flowering; *right photo*, detail of flower buds arising directly from the stem

1.0 unit of Taq polymerase (Invitrogen), 0.3 μM of both primers, and 6.0 ng of genomic DNA. A hot start PCR program was used with a 5-min denaturation step followed by 29 cycles of 1 min at 94°C, annealing for 1 min with temperatures between 48 and 65°C according to the marker locus and extension for 1 min at 72°C. PCR was carried out for each marker individually, and 1 μl of PCR product from each of three markers with different fluorescences (6-FAM, NED, and HEX) were pooled and mixed with 1 μl of ROX size standard [6] and 10 μl of Hi-Di formamide (Applied Biosystems). The mixture was electroinjected in an ABI 3100 genetic analyzer, and data collected under virtual filter D using Genescan (ABI) and analyzed with Genotyper (ABI).

Identification of microsatellite markers linked to the early flowering locus A BSA approach [25] was used to identify microsatellite markers linked to the early flowering phenotype. Within the (A0085×U0243) segregating progeny of 88 plants, the DNA of 15 early flowering plants and 15 wild-type plants was extracted individually and mixed in equimolar quantities. Thus, two DNA bulks, one from early flowering plants (called EF bulk) and one from wild-type not flowering plants (called NF bulk), were obtained. The two DNA bulks were screened with 100 EMBRA microsatellites distributed along the reference map of *E. grandis* [4, 5] and previously screened for polymorphism between parents A0085 and U0243. BSA using microsatellite markers was carried out by comparing the fluorescence intensity in relative fluorescence units (rfu) of contrasting alleles segregating from the mutant parent. Relative differences of more than 100% were considered as evidence of linkage of the marker with the phenotype. To avoid false positives, markers showing rfu differences among alleles inherited from the mutant parent were genotyped three times before declaring it as a putatively linked marker. These markers were then typed in the full segregating progeny to verify linkage and estimate recombination fraction. For map construction the software, JoinMap [29] was used with thresholds of θ=0.4 and LOD=8.0. For marker ordering, the ripple command was used after each marker was added.

Results and discussion

The BSA using microsatellite markers was successful in identifying markers linked to a major QTL for early flowering in *E. grandis*. Most searches for major effect loci using BSA have been carried out with dominant random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) markers. These classes of markers have the advantage of assaying a much larger number of polymorphic sites in the target genomic region therefore increasing the probability of finding closely linked markers. For example, in *E. grandis*, this approach revealed a RAPD marker cosegregating without a single recombinant in 965 meioses with a *Puccinia* rust-resistance locus [22]. BSA and dominant markers have been successfully used to map QTL for disease-resistance loci
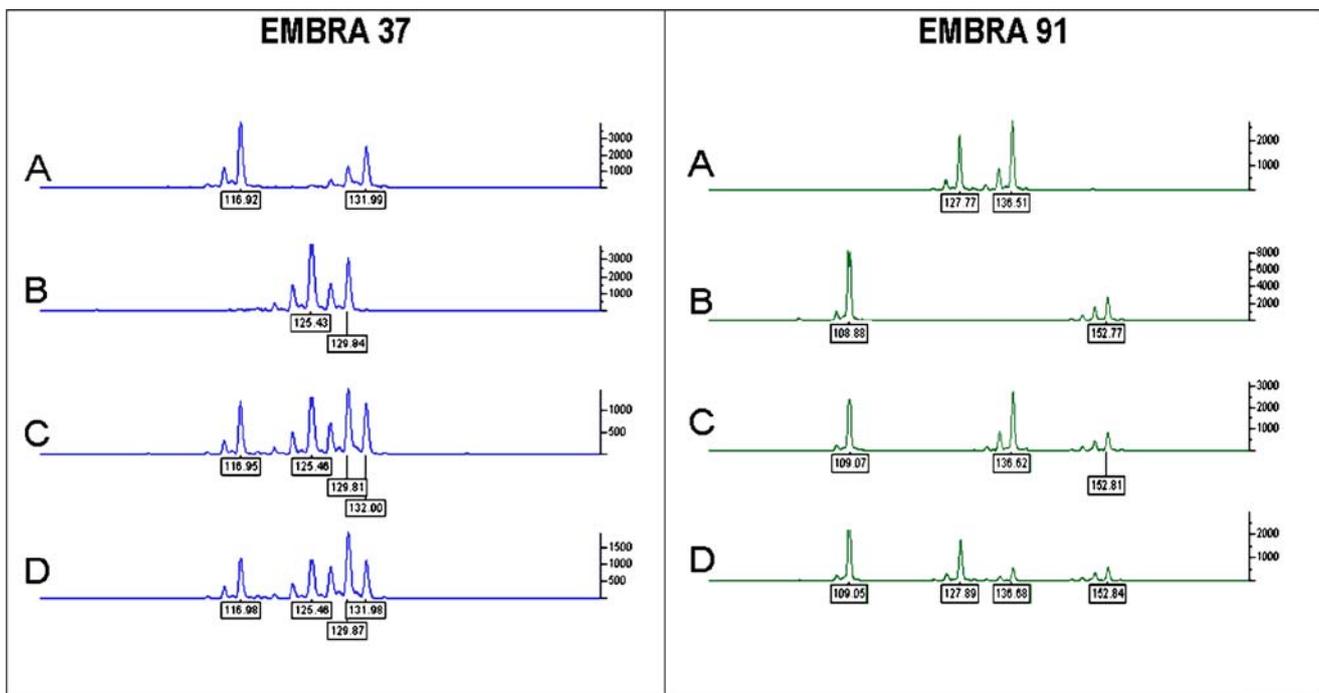

Fig. 2 BSA with microsatellite markers. From *top* to *bottom*, electropherograms showing the mutant parent alleles (**a**), the wild-type parent (**b**), the early flowering bulk (**c**), and the no flowering bulk (**d**). *Left*, EMBRA37 marker is not linked to the *Eef1* QTL as the mutant parent alleles display similar fluorescence intensity in the two bulks. *Right*, EMBRA91 alleles, on the other hand, display a clear difference in fluorescence intensity between the two bulks with allele 136,32 (rounded up to 137) linked in coupling to the mutant phenotype allele
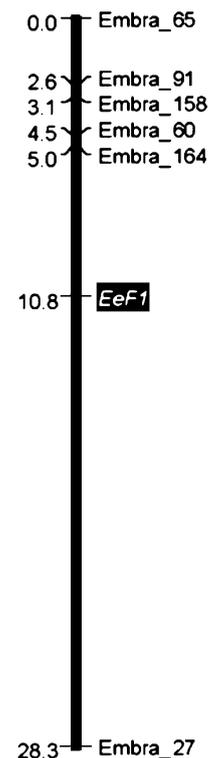
Table 1 Microsatellite markers linked to the *Eef1* QTL and results of their full cosegregation analysis with the early flowering phenotype

Microsatellite marker	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Alleles (pb)	Early flowering (*n*)	No flowering (*n*)	LOD score for linkage to *Eef1*	Distance from *Eef1* (Kosambi, cM)	Allele in coupling with *Eef1* (bp)
EMBRA27	ataaccacaccaatctgca	tatagctcgaacgctcaac	136	4	34	5.64	21.80	153
			153	28	12			
EMBRA60	aacagcagttgctacaccac	gagcgaaaaggagaacacc	106	1	46	15.80	6.28	127
			127	29	4			
EMBRA164	ccttgttgagctcctgtct	actatcagcgtcctgcaa	133	32	5	16.57	6.44	133
			135	0	41			
EMBRA65	gacatctcctcctcaagc	cgatatgctacgtcttcc	209	0	43	13.81	10.88	Null
			Null	32	9			
EMBRA158	gtgcagatatcaccacct	cattcagttcccagtacc	141	1	24	8.21	9.36	151
			151	25	4			
EMBRA91	tgttcctggattgtcactta	atccagattgagcacagac	128	1	41	15.35	7.37	137
			137	33	5			

in forest trees such *Populus deltoides* [7, 32], *Pinus lambertiana* [9], *P. taeda* [34], and *Ulmus parvifolia* [3]. Although powerful for fine mapping, such dominant markers might not segregate in other crosses involving the same individual requiring conversion to a sequence-specific marker. Furthermore, these sequence-specific markers rarely can be transferred across different pedigrees hindering their use for preliminary testing of allelism between genomic segments containing QTLs for the same trait. This should be particularly critical in outbred genetically heterogeneous forest trees. BSA with microsatellites, on the other hand, requires a large numbers of markers upfront to allow screening a sufficient set of polymorphic ones and thus finding close linkages. However, once microsatellite markers are found linked to the target locus, they can be efficiently used to transfer this information to reference maps and unrelated pedigrees and used as starting points for positional cloning efforts. With a more extensive availability of microsatellite markers in several crop plants, these markers have increasingly been used in BSA experiments [1, 2, 27].

Of the 100 screened microsatellite markers, six of them displayed a clear contrast in relative fluorescence intensity between the two alleles inherited from the mutant parent tree (Fig. 2). The putative linkage of these six markers was further validated in the segregating progeny set and mapped around the *Eef1* QTL (Table 1 and Fig. 3). Not all 88 plants were available for genotyping, therefore, the effective number of plants genotyped varied from marker to marker with a minimum of *n*=54 for EMBRA158 to a maximum of *n*=84 for EMBRA65. The six markers did segregate in a 1:1 ratio as evaluated by a chi-square test. The flowering phenotype, however, displayed segregation distortion from the expected 1:1, with a slightly larger number of plants not expressing the early flowering phenotype (Table 1). This could be due to incomplete penetrance or variable expressivity of the phenotype at evaluation age. Three of these marker loci, EMBRA27,

EMBRA60, and EMBRA65, were previously published and mapped to linkage group #2 [5]. The other three markers, EMBRA91, EMBRA158, and EMBRA164, were also mapped in a consensus linkage map constructed with 206 microsatellites [4]. Marker ordering between the localized linkage map around *Eef1* and the reference map of Brondani et al. [5] is perfectly conserved with markers EMBRA65 and EMBRA60 closer together, followed by EMBRA27 at a larger distance over 20 cM. When compared to the higher coverage linkage map of Brondani [4], the order is again conserved involving markers EMBRA65, EMBRA60, EMBRA164, and EMBRA27. Closely linked

Fig. 3 Linkage map of microsatellites around the *Eef1* QTL

markers EMBRA91 and EMBRA158, however, map between markers EMBRA65 and EMBRA60 in the *Eef1* map, but map between EMBRA60 and EMBRA164 in the consensus map of Brondani [4]. These two markers do not improve the linkage information to localize *Eef1* as this QTL is flanked by EMBRA164 and EMBRA27. They could be useful, however, in unrelated early flowering pedigrees when the two flanking markers are not informative. The conservation of marker order around *Eef1* in unrelated pedigrees, and the fact that it was mapped between highly transferable microsatellite markers, will allow future comparative mapping analyses and tests of QTL allelism between the genomic segment containing *Eef1* and other QTL mapped from independently discovered mutants for early flowering.

None of the other 94 microsatellite markers tested showed evidence of linkage with early flowering in the BSA experiment. Nevertheless, the identification of a major effect QTL in a BSA approach does not preclude the existence of other loci of minor effect that BSA will not detect. Grattapaglia et al. [16] in a sequential QTL-mapping approach found that BSA detected one of the three QTL for *Eucalyptus* growth later found by a full scale cosegregation analysis. In this work, the early flowering phenotype was measured by a binary score—flowering × not flowering—with a strict operational breeding perspective. No refined measurements such as days to flower from germination and number of flower buds were assessed. Nonetheless, within the early flowering plants, variable expressivity was observed, with some plants flowering earlier at around 60 days and some others later, after 100 days. These results taken together suggest that *Eef1* is responsible for the largest part of the expression of this early flowering phenotype, but that other loci most likely exist also involved in the control of trait expression.

Introgression of the early flowering dominant allele at *Eef1* to other trees, could be monitored with flanking markers EMBRA164 (at 6.44 cM) and EMBRA27 (at 21.8 cM) with an estimated efficiency of 98.6% (0.064× 0.218=0.014), assuming no recombination interference in the target genomic segment. It is true, however, that one could easily verify the desired early flowering plants just by waiting for its expression at age 90 days. It will be in fact interesting to verify the expression of this dominant mutation in different genetic backgrounds, both in the same *E. grandis* species, as well as in later flowering ones such as *E. globulus* and *E. dunnii*, both sexually compatible with *E. grandis*. We have started such experiments by crossing A0085 to a few other *E. grandis* and *E. urophylla* trees and in all five progenies obtained, the early flowering phenotype was observed segregating in a mendelian fashion (R. Medeiros, personal communication).

To follow into a positional cloning effort of *Eef1*, more closely linked markers would be needed. We did test a battery of 450 RAPD markers that revealed about 2,000 bands, but we could not find a single robust RAPD marker closer to *Eef1* than the microsatellites mapped (N. Bueno, unpublished data). Besides a molecular screening tool that uncovers a larger numbers of polymorphisms in the *Eef1*

region, a much larger progeny size would be necessary to find flanking markers that fully cosegregate in several hundred meiotic events. With a relatively modest genome size of 640 Mbp for *E. grandis* [14], and being the early flowering phenotype an easy one to score, a positional cloning effort should prove successful. A concurrent approach could be the tentative colocalization of candidate genes with *Eef1* by genetic mapping using high throughput technologies. Candidate genes could be tested, rationally chosen from the fast growing databases of *Eucalyptus* ESTs [13] and functional genetics information from *Arabidopsis* as well as other model plant species.

In conclusion, we have identified and genetically mapped an early flowering locus derived from a naturally occurring mutant tree of *E. grandis*. This report corroborates the fact that the relatively undomesticated condition of *Eucalyptus* species represents an open field for the exploration of natural genetic variation. This has already been true for growth, adaptability, and wood quality traits, generating substantial genetic gains by breeding in the last 30 years. It is important to point out that, besides the early flowering phenotype, mutant *E. grandis* A0085 not only has normal growth but it stands out as a selected tree. Besides its potential use in breeding practice, A0085 could be very useful for experimental genetics and genomics of *Eucalyptus*. Inbred lines development, transgene technology, and functional genomics studies could certainly benefit from the use of this and other early flowering mutants.

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