

Growing up fast: manipulating the generation time of trees

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Domestication and genetic improvement of trees is far behind that of herbaceous plants owing to their long generation times, which result from the existence of a long juvenile phase of reproductive incompetence. During recent years, significant progress has been made towards understanding the molecular basis of flowering transition in model herbaceous species. Some of the genes identified have been shown to efficiently accelerate reproductive development when ectopically expressed in transgenic plants, including trees. These results provide new clues as to the molecular basis of reproductive competence in trees and suggest ways to accelerate their genetic improvement.

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Current Opinion in Biotechnology 2002, 13:151–155

0958-1669/02/\$ – see front matter

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Abbreviations

LD long day
 SAM shoot apical meristem

Introduction

Economically important woody species such as fruit and forest trees have undergone little domestication and breeding compared with herbaceous crop species. Many forest tree cultivars only represent F₁ or F₂ generation genotypes that are vegetatively propagated [1]. The main reason for this delay in the genetic improvement of woody species can be traced to their long generation times. Additional difficulties of tree breeding, such as inbreeding depression, could be overcome with the availability of rapid cycling lines that would accelerate the selection of inbred lines with reduced genetic load.

Adult trees behave as perennial plants that in many cases flower seasonally every year. After germination, however, young tree seedlings are generally unable to initiate reproductive development and require a period of shoot maturation known as the juvenile phase. Tree generation time is equivalent to the duration of this juvenile phase, which is species-specific and can last from one year to several decades [2]. Approaches to reduce tree generation time should focus on reducing or even suppressing this juvenile period to accelerate the acquisition of reproductive competence. Current understanding of the control of flowering transition is mostly based on the study of a single herbaceous annual species, *Arabidopsis thaliana* [3–5].

Results are far from explaining the molecular basis of reproductive competence in annual or perennial species. However, the reduction in tree generation time achieved by the ectopic expression of some *Arabidopsis* genes have fostered interest to understand how reproductive competence is regulated in trees and to develop tools to promote it. This report summarises the advances in characterising the regulation of flowering transition in tree species, highlighting the approaches used to accelerate the generation time of trees.

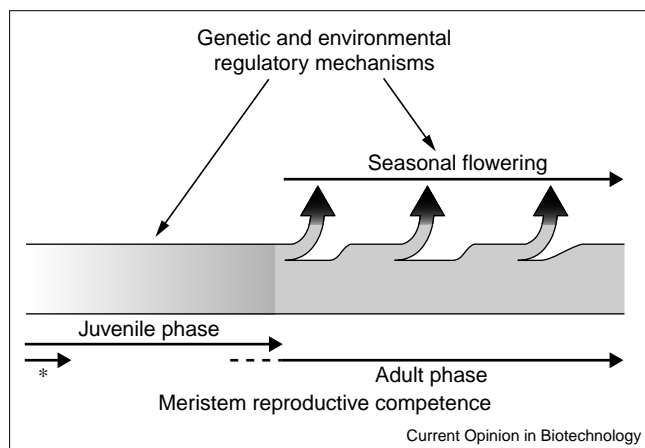
Plant juvenility

Plant post-embryonic development is the result of mitotic activity at the shoot apical meristem (SAM). The SAM is formed by undifferentiated cells the division of which generates lateral primordia that give rise to all the aerial organs of the plant. Upon flower induction, the SAM and/or derived lateral meristems change their fate to generate inflorescence or flower meristems, developing reproductive structures. All higher plants are unable to initiate reproductive development immediately after germination and have to undergo a process of shoot maturation, or the juvenile developmental phase. During this phase meristems acquire reproductive competence, becoming able to sense and respond to signals that induce flowering [2,6]. Perennial plants, such as trees, which have to reach a threshold size for being competent in reproduction, generally display long juvenile phases [6]. Even fast-growing herbaceous annual plants avoid flowering immediately after germination and display a short juvenile phase of reproductive incompetence [7,8]. Juvenility is recognised in many tree species by morphological, cellular or physiological traits, such as specific leaf size and shape, presence of stem thorns or the ability to root, which are lost in the adult phase [2]. In a herbaceous annual species, such as *Arabidopsis*, the juvenile phase has been related to the production of round small leaves lacking abaxial trichomes [7,8]. Acquisition of reproductive competence takes place gradually in parallel with the disappearance of juvenile traits. Adult plants continue to grow vegetatively but, in contrast to juvenile plants, will initiate flowering whenever the environmental conditions are favourable.

Genetic and environmental control of the juvenile phase

The lack of genetic tools and the long generation time make it difficult to analyse the genetic control of reproductive competence in trees. Genetic variation for the length of juvenile phase has been described in several woody species [6]. In birch, this variation has allowed the selection of rapid cycling lines that are competent to flower in their first year of growth instead of the 5–10 years usually required by birch seedlings [9]. Natural variation for the length of juvenile phase has not been analysed in

Figure 1



Schematic representation of meristem reproductive competence along shoot development of trees. An increase in meristem reproductive competence is represented by darker shading. Initially, all juvenile meristems are reproductive incompetent (white region, marked with an asterisk). As the tree grows, new juvenile meristems, which still produce juvenile shaped organs, show increased capacity to respond to extreme flowering inducing conditions (increasing grey region). Adult phase meristems are reproductive competent (uniform grey region); however, only a set of adult meristems will acquire seasonal reproductive-competence and initiate flowering every year or whenever environmental conditions are favourable. Both, the juvenile to adult transition and the seasonal induction of flowering could be controlled through common genetic and environmental regulatory pathways.

herbaceous species; however, several loci affecting this process have been identified owing to spontaneous or artificial mutations in maize, pea and *Arabidopsis* [2]. The most extensive genetic analysis has been performed in *Arabidopsis* on the basis of the presence of leaf abaxial trichomes [10]. Mutations that specifically prolong the juvenile phase have not been identified. However, mutations that shorten the juvenile phase without causing major effects in the length of other developmental phases have been isolated at several loci including *HASTY*, *PAUSED* [8,10], *SERRATE* [11,12*], and *SQUINT* [13**]. Molecular identification of some of these loci has shown that *SERRATE* encodes a zinc-finger putative nuclear protein [12*], whereas *SQUINT* encodes a cyclophilin 40 homologue [13**]. Furthermore, most mutations that accelerate or delay the flowering transition of adult plants also shorten or prolong the juvenile phase, respectively [7,8,10].

Acquisition of reproductive competence is highly dependent on environmental cues, such as photoperiod and temperature, which induce flowering in adult plants. These responses have not been fully analysed in trees because of the difficulties of growing trees in controlled environmental conditions. However, growing conditions have drastic effects on tree reproductive competence. For instance, birch plants grown under continuous and intense illumination, in high CO₂ rooms, are competent to flower 3–5 months after germination instead of the 5–10 years

normally required [14]. Similarly, olive tree plants grown in the greenhouse at high temperature and continuous light, and adequately pruned, initiate flowering after 2–3 years instead of the 10–15 years usually required [15]. Reproductive competence is also accelerated or delayed by environmental conditions in herbaceous species such as *Arabidopsis*. Both long photoperiods and vernalisation (i.e. low-temperature exposure), which induce flowering in adult plants (see below), also reduce the length of the *Arabidopsis* juvenile phase.

The information available on herbaceous and woody species suggests that acquisition of reproductive competence is under genetic and environmental controls. The fact that, in *Arabidopsis*, mutations and environmental conditions that delay or accelerate flowering in adult plants also affect the juvenile phase in the same direction, opens the question of whether an independently controlled acquisition of reproductive competence really exists [7,8,16]. Additionally, the absence of complete suppression of the juvenile phase in the above mentioned examples suggests the existence of an initial period of absolute reproductive incompetence followed by a gradual acquisition of competence to respond to environmental cues [10,11]. Thus, flower initiation would result from the balance between reproductive competence of the meristem and the strength of the flowering inducing factors (Figure 1). This would explain why drastic changes in environmental conditions can make trees initiate flowering.

Flowering transition in adult plants

Once plant meristems are reproductively competent the adult state is maintained through cell division throughout the life of the plant [17]. In annual plants, all shoot meristems reach competence within one year and most would produce flowers before senescence of the plant. By contrast, in adult trees only a portion of the shoot meristems will initiate flowers each year, suggesting different levels of reproductive competence among meristems (Figure 1). This differential competence ensures flowering and seed production in following years. Whether the mechanism regulating competence in adult tree meristems is the same as that responsible for the acquisition of competence in the juvenile to adult transition remains to be elucidated.

Environmental cues, such as photoperiod, light intensity, temperature or water availability, that induce flowering in adult trees are species-specific. The genetic and molecular mechanisms governing these flowering responses in trees are mostly unknown. In adult vegetative plants of *Arabidopsis*, flowering is accelerated by long photoperiods and by the exposure to low temperatures in vernalisation. Consistent with these environmental requirements, two main flowering induction regulatory pathways have been identified: the long day (LD) and autonomous pathways [18]. The LD or photoperiod pathway promotes flowering under inductive long photoperiods. It involves the function of photoreceptors, such as phytochromes and cryptochromes, and ends with the

transcriptional activation of genes such as *FT* and *SOC1* [4]. The FT protein shows certain similarities to phosphatidylethanolamine-binding proteins [19,20], whereas *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CO1*) encodes a MADS-box transcription factor [21••,22••]. The autonomous pathway promotes flowering independently from photoperiod and its function converges with vernalisation. Both the autonomous pathway and vernalisation treatment reduce the expression of *FLC* (*FLOWERING LOCUS C*) [23], another MADS-box gene that functions as a flowering repressor [24,25]. *FLC* has a negative effect on the expression of *FT* and *SOC1* [22••]. Thus, *FT* and *SOC1* integrate signals from both pathways to promote the activation of flower meristem identity genes such as *LFY* (*LEAFY*) and *API* (*APETALA*) and the initiation of flowering. *LFY* encodes a unique transcription factor in *Arabidopsis* [26] and other angiosperms [27]. *API* encodes another MADS-box gene [28] and its function is partially redundant with that of other MADS-box genes such as *CAL* (*CAULIFLOWER*) [29] or *FUL* (*FRUIT-FULL*), which also have a role in flower meristem specification [30].

Overexpression of genes participating in the LD pathway [21••,31•], the autonomous pathway [32,33,34] or of *FT* and *SOC1* [19,20,22••], cause acceleration of flowering in *Arabidopsis*. Similar effects have also been observed when overexpressing flower meristem identity genes such as *LFY*, *API* or *FUL* [30,34,35] or even MADS-box flower organ identity genes, such as *AG* (*AGAMOUS*) or *SEP3* (*SEPALLATA 3*) [36,37]. Whether all these effects on the acceleration of flowering result from the rapid acquisition of reproductive competence or are only effective on adult plants has not been analysed in all cases. The only reported results suggest that expression of *FT*, *SOC1* or *API* [19,20,21••,22••,35] accelerate the acquisition of reproductive competence, whereas expression of *LFY* does not [34].

LFY-like genes have also been isolated from angiosperm and gymnosperm tree species [38,39,40•,41] and their capabilities to promote flowering have been shown by ectopic expression in transgenic *Arabidopsis* [38,39,40•]. Similarly, *API* orthologues have been identified in eucalyptus [42], apple trees [43] and birch [44] and their effects on flower initiation shown in transgenic *Arabidopsis* or tobacco [42–44]. However, the lack of efficient transformation systems and the long time involved in the regeneration and flowering process have prevented a demonstration of the function of these genes in their respective species.

Manipulation of generation time in trees

In 1995 Weigel and Nilsson demonstrated that constitutive expression of the *Arabidopsis* gene *LFY* in a male hybrid aspen clone (*Populus tremula* × *Populus tremuloides*) induced the development of flowers in transgenic juvenile trees, which would normally take 15 years to become competent to flower [34]. These results showed that tree juvenile phase could be reduced through genetic engineering. Unfortunately, these transgenic aspen shoots developed

abnormal terminal flowers and single male lateral flowers that did not shed pollen. Later reports [40•] have shown that competence to respond to the constitutive expression of *LFY* varies widely among different interspecific *Populus* hybrids, and only gives consistent early flowering in *P. tremula* × *P. tremuloides* hybrid clones. *API* was also constitutively expressed in hybrid aspen, but did not have any effect [45]. Other *Arabidopsis* flowering time genes such as *CO* (*CONSTANS*) and *FCA*, respectively involved in the LD and autonomous regulatory pathways, have also been expressed in hybrid aspen, but did not have any flowering promoting effect (O Nilsson, personal communication).

More recently, *LFY* and *API* have been expressed in citrange, a commonly used citrus hybrid root-stock (*Poncirus trifoliata* × *Citrus sinensis*) [46••]. When grown from seed these trees take 6 to 7 years to become reproductively competent; however, constitutive expression of either *LFY* or *API* drastically reduced the length of the juvenile phase and accelerated reproductive competence. Thus, transgenic trees initiated flowering after 12–18 months of growth, corresponding with the spring season. Flowers were regular and fertile in both transgenic types. Transgenic trees expressing *API* displayed normal development as adult trees. By contrast, ectopic expression of *LFY* produced alterations in leaf and stem development and a tendency to initiate single terminal flowers. Interestingly, expression of *LFY* or *API* did not result in flower formation in all meristems of the plants, indicating the existence of endogenous competence controls that were not overcome by the expression of the transgenes. Furthermore, transgenic trees were still able to respond to the environmental cues that induce flowering every year [46••]. The fertility and short generation time of these transgenic trees allowed a demonstration of the inheritance of the new trait both in apomictic and zygotic descendants. These results open the door for future applications of transgenic lines as rapid cycling breeding lines.

Getting incompetent meristems to flower

How does constitutive expression of *LFY* or MADS-box genes reduce the length of the juvenile phase and promote reproductive competence of tree meristems? In other words, how does the expression of these genes cause an incompetent meristem to flower? In *Arabidopsis*, constitutive expression of *LFY* is unable to overcome meristem incompetence to flower [34], in contrast with the effects of *LFY* in transgenic trees [45,46••]. It is hard to compare *LFY* effects in a herbaceous species, with a juvenile period of a few days, with its effects on a woody species with several years of juvenility. Still, if we assume that *LFY* has no effect on meristem competence, on the basis of the phenotype of *Arabidopsis 35S::LFY* transgenics, then we will have to propose that, in trees, there is a gradual increase of meristem competence along the development of the juvenile phase. Thus, *LFY* may cause flowering at suboptimal competence levels, an effect not observed in *Arabidopsis* because of its short juvenile phase. In addition

to this developmental control of competence, there should be an environmental control. In *Arabidopsis* this environmental control could be responsible for the delay in flowering caused by short days on *35S:LFY* transgenic plants, whereas in *Citrus* it could control flower seasonality. Under this hypothesis, the differential effects of *LFY* expression in several tree genotypes could be explained by variation in meristematic competence, which could possibly be affected by growing the transgenic trees under various environmental conditions.

The suppression of the juvenile phase by constitutive expression of MADS-box genes requires additional considerations. There are a large number of MADS-box genes in plants [47]; MADS-box proteins work as homodimers or heterodimers displaying different DNA-binding affinities and activation capabilities [48]. This makes the effect of the expression of a MADS-box gene in a heterologous species highly unpredictable. In contrast to *LFY*, constitutive expression of *API* in *Arabidopsis* has been shown to partially reduce the length of juvenile phase [10]. Furthermore, suppression of juvenile traits in transgenic *Citrus* is more evident in *35S::API* than in *35S::LFY* plants [46**]. As proposed for *LFY*, we cannot discard a role for *API* in promoting the specification of flower meristems in suboptimal competent meristems. However, *API*, and perhaps other MADS-box proteins, might interact or interfere with other MADS-box genes more specifically involved in regulating the acquisition of reproductive competence. Differential interactions in heterologous species would explain the distinct effects observed in *Populus* and *Citrus*.

Conclusions and perspectives

Tree generation time can be reduced through genetic manipulation, although similar genes can have different effects in different species. The lack of information about the molecular mechanisms regulating meristem reproductive competence in trees, and the scarcity of experimental results in a broad range of species, prevent us from making any general conclusion. Progress in understanding the regulation of meristem competence and flower initiation in *Arabidopsis* will provide additional gene sequences to be tested in trees. Furthermore, strategies to reduce the juvenile phase will have to be refined to avoid unwanted effects on plant development. Although juvenile phase suppression can accelerate the breeding of tree varieties, it may also affect the development of the tree and reduce its final production. Strategies to eliminate the transgene once a new variety has been developed will have to be considered, as simple genetic segregation will not be efficient in highly heterozygous tree species. Several approaches involving the activation of site-specific recombinases [49] can be useful to remove transgenes after breeding.

Different genes and gene constructs could be useful to reduce juvenility in several tree species; however, these approaches would only represent trial and error experiments unless further understanding of the mechanisms

governing reproductive competence is gained in trees. Hopefully, rapid cycling trees will not only help to accelerate breeding in economically important species, but may constitute useful experimental tools as laboratory strains for functional genetic analyses of tree biology. Full domestication of fruit and forest trees can now be seen to be a little bit closer.

Acknowledgements

We thank Ove Nilsson for sharing unpublished results. We also thank Carlos Alonso, Pilar Cubas, José A Jarillo, Ove Nilsson and Manuel Piñeiro for critical reading of the manuscript. Our work is supported by grants AGF98-0206 and BIO01-3891 from Comisión Interministerial de Ciencia y Tecnología (CICYT), Spain and QLK5-CT-2001-01412 from the European Union. Support to research activity at Centro Nacional de Biotecnología is provided through a specific agreement CSIC-INIA.

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