Review article

Mejoramiento genético acelerado de angiospermas perennes vía inducción floral por sobre-expresión del gen *FT*

Accelerated genetic improvement of angiosperm perennial species via floral induction by over-expression of the *FT* gene

Rafael Urrea-López[1]

Resumen

Los bosques y selvas enfrentan el reto de satisfacer la demanda por recursos de una población en crecimiento, así como la amenaza del rápido cambio climático que exacerba la magnitud y frecuencia de estreses bióticos y abióticos. Para ello, es urgente acelerar el mejoramiento genético de especies forestales. Sin embargo, sus largas etapas juveniles y asincronía floral retrasan peligrosamente este proceso. El presente ensayo explora los adelantos biotecnológicos en inducción floral y su potencial aplicación en especies forestales. Entre los genes identificados y caracterizados que participan en la ruta de señalización de la floración, especial atención se destina al gen *FLOWERING LOCUS T*, considerado un integrador de rutas de señalización altamente conservado entre las angiospermas, que, al sobre-expresarse por ingeniería genética, es capaz de inducir la floración de forma eficiente. Esta novedosa estrategia biotecnológica se ha utilizado, recientemente, para segregar genes de resistencia a enfermedades, en un menor tiempo, en germoplasma comercial de manzana y ciruela. Permite soslayar barreras naturales que por mucho tiempo han restringido a las especies forestales al mejoramiento por selección, principalmente. Entre sus ventajas está la de poder restringirla al proceso y no al producto, para acelerar las cruzas sexuales sin modificar genéticamente la progenie; se aleja así de la controversia alrededor de la liberación y consumo de organismos genéticamente modificados, y de los costos y trámites obligatorios para los OGM para monitoreo de posibles riesgos. Se proyecta como una tecnología que puede acelerar, significativamente, el mejoramiento de especies forestales.

Palabras clave: Biotecnología forestal, *FLOWERING LOCUS T*, ingeniería genética vegetal, mejoramiento acelerado de perennes, mejoramiento de árboles, inducción de floración.

Abstract

Forests and tropical forests face the challenge of meeting the demand for resources from a growing population, as well as the threat of rapid climate change that exacerbates the magnitude and frequency of biotic and abiotic stresses. For this, it is urgent to accelerate the genetic improvement of forest species. However, its long juvenile stages and floral asynchrony dangerously delay this process. This essay explores biotechnological advances in floral induction and its potential application in forest species; among the identified and characterized genes involved in the flowering signaling path, special attention is given to the *FLOWERING LOCUS T* gene, considered an integrator of highly conserved signaling pathways among angiosperms, which, when over-expressed by genetic engineering, it is able to induce flowering efficiently. This innovative biotechnological strategy has recently been used to segregate disease resistance genes, in a shorter time, in commercial apple and plum germplasm. It allows to avoid natural barriers that have long restricted forest species to breeding by selection, mainly. Among the advantages of this strategy is to be able to restrict it to the process and not to the product, to accelerate the sexual crossings without genetically modifying the progeny; it moves away from the controversy surrounding the release and consumption of genetically modified organisms, and the costs and obligatory procedures in GMOs for monitoring possible risks. It is projected as a technology that can significantly accelerate the improvement of forest species.

Key words: Forest biotechnology, *FLOWERING LOCUS T*, plant genetic engineering, accelerated tree breeding, forest tree breeding, flowering induction.

[1]Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C. Unidad Noreste. México. Correo-e: rafaelurrea80@yahoo.es

Population growth and forest resources

The world population is experiencing continuous growth and by 2050 is projected to increase 30 % to add more than 9 000 million people, an increase that translates into a greater demand for natural resources to meet their needs (FAO, 2014). The Food and Agriculture Organization of the United Nations (FAO) estimates that the demand for timber and energy resources for industrial and domestic uses will increase by 40 % by the middle of 2030.

The growth of the demand for forest resources has driven the selective extraction of wood and the deforestation of forests and natural forests. In the first decade of the 21st century it is estimated that 40 million hectares of primary forests were lost worldwide (FAO, 2010).

Climate change and forest resources

Currently, at the same time as the demand for forest resources increases due to a growing population, environmental changes of great magnitude are experienced due to climate change, as a result of the accumulation in the atmosphere of anthropogenic emissions of greenhouse gases (carbon dioxide, nitrous oxide and methane, mainly) since the pre-industrial era. Rapid global warming causes alterations in the intensity and frequency of climatic phenomena such as droughts, torrential rains and floods; these conditions can cause biochemical, molecular, physiological and morphological changes in plants, which depending on the intensity and duration as well as the stage of development of the plant, have significant consequences in their growth, development and reproduction. This limits the expression of their genetic yield potential and affects the geographical distribution of the species by the desynchronization with the climate to which they adapted (Doroszuk *et al*., 2006; Srinivasa *et al*., 2016).

Additionally, climate change can exacerbate biotic threats, due to the effects on the biology, physiology and dynamics of populations, as well as the ecological relationships between pathogens with the other biota in their ecosystem (Garrett *et al.,* 2006).

The direct effects (precipitation, temperature), and indirect effects (increase of pests, diseases and fires) of climate change can significantly impact the conservation of biodiversity and, therefore, the provision of goods and services to meet the needs of a population worldwide increase (UN-DESA, 2015), mainly of those plant species with longer biological cycles than the others, as is the case of perennial species.

Loss of biodiversity of forest resources

Climate change causes anomalies in the phenology of perennial species, such as a lower rate of germination of floral and vegetative shoots, a less vigorous growth of the shoots and a lower fixation and filling of the fruits (Ramírez and Kallarackal, 2015).

Illegal logging along with biotic and abiotic threats exacerbated by the effects of climate change pose a threat to the biodiversity of ecosystems by affecting the sizes and structures of populations and diminishing the diversity and genetic richness of their species, which induces to the extinction of the species.

Globally there are already significant impacts on the forest resources associated with this factor, such as the massive death of *Pinus edulis* Engelm. in 12 000 km^2 at south-western United States (Breshears *et al*., 2005); a reduction of poplar (*Populus tremuloides* Michx.) in western United States (Rehfeldt *et al.,* 2009), of cedar (*Cedrus atlantica* (Endl.) Manetti ex Carrière) in Morocco, and of common beech (*Fagus sylvatica* L.) in south-western Hungary (Mátyás, 2010).

The speed at which this problem reveals itself exceeds the ability of the trees to face it, which propitiates a reduction in their resistance and recovery from extreme weather events, and / or the attack of pests and diseases, which represents a serious risk for the preservation of the genetic diversity of many species (Jump and Peñuelas, 2005).

The different challenges and threats that climate change imposes on forest resources urgently demand accelerating the genetic improvement of forest species

with greater yield and tolerance to biotic and abiotic stress conditions. However, perennial species face the same challenge in particular, the extensive period of the juvenile stage before flowering, which in angiosperms is usually between seven years or more (Häggman *et al.,* 2013).

FT gene in the regulation of flowering in angiosperms

Recently, scientific and technological advances in genomics have helped to reveal the biological function of genes present in plants. Its functional characterization is carried out through the deliberate manipulation of the positive or negative expression of one or several of them, for which the sequencing of the genome and the use of model systems is indispensable.

The first sequenced plant genome was that of *Arabidopsis thaliana* (L.) Heynh. sequenced in the year 2000. This plant is currently the most widely used model system, due to its small size, short life cycle, high production and seed viability, ease of crossing by autogamy, and small genome (<200 megabases) (The Arabidopsis Genome Initiative, 2000).

At present, angiosperms constitute the most diverse group of terrestrial plants, since around 79 % of the 374 000 plant species described correspond to this phylum (Christenhusz and Byng, 2016). They are vascular plants with tissues and organs perfectly differentiated; its most outstanding characteristic and key factor of its evolutionary success is its complex reproductive structure (flower), which gives rise to the production of seeds covered by a fruit.

The model plant *Arabidopsis* has served enormously for the study of the flowering process, in which the differentiation of the apical meristem to floral meristem is regulated by genetic, epigenetic and environmental factors. Among the main stimuli that control the transition from vegetative to reproductive stage in *Arabidopsis* are the endogenous stimuli (autonomous pathways, gibberellin, circadian clock, age, sugar levels) and environmental (vernalization, room temperature and photoperiod), organized in complex and

hierarchical signaling routes. Both the routes triggered by the internal state of the plant and in response to exogenous factors, converge in flowering integrating genes that activate meristem identity genes, among them the *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*); *FLOWERING LOCUS T* (*FT*) and *AGAMOUS-LIKE24* (*AGL24*) (Blümel *et al.,* 2015).

The transition through the different life cycles in plants is regulated transcriptionally and post-transcriptionally by short sequences of non-coding RNAs (miRNA) that affect the expression of key transcription factors. In the case of the transition from juvenile to adult stage, miR156 negatively controls the expression of 11 of the 17 transcription factors *SQUAMOSA PROMOTER BINDING-LIKE (SPL)*, which affects, in turn, the expression of the integrative gene of the flowering *FT* route (Spanudakis and Jackson, 2014).

In *Arabidopsis*, the transcription of *FT* is promoted by the transcription factor *CO* (CONSTANS), which is governed by the biological clock of the plant when exposed to more than 12 hours of light, during long days (Calviño *et al.,* 2005). The *FT* gene is highly conserved among angiosperms; it participates in the transition to flowering in neutral plants to the extension of the day as tomato (Lifschitz *et al.,* 2006) and banana (Chaurasia *et al.,*2017), as well as in plants that require vernalization (Yan *et al.,* 2006), for which it is considered as an integrator of signaling routes.

Once the conditions that activate the transition from the vegetative to the reproductive phase are perceived by the plant, the expression of the *FT* gene in the vascular tissues of the leaves is triggered. The resulting *FT* protein (~ 20 KDa), from the family of phosphatidylethanolamine binding proteins (Kardailsky *et al.*, 1999), is transported through the phloem to the apical meristems. It stands out as one of the few examples of macro-molecules in plants that travel great distances to play a role in receptor tissues (Abe *et al.,* 2015; McGarry and Kragler, 2013). Once at the apex of the outbreak, the *FT* protein interacts with the transcription factor *FLOWERING LOCUS D* (*FD*). The *FT / FD* heterodimer triggers a cascade of positive transcriptional signals that activate several transcription factors of genes such as *APETALA1* (*AP1*)*, FRUITFULL* (*FUL*)*, SOC1,* and *LEAFY* (*LFY*) that lead to the

reprogramming of the primordium to produce reproductive organs in instead of vegetative (Abe *et al.*, 2005; Teper and Samach, 2005).

Additionally, *FT* type proteins have been related as regulatory factors in a large number of development processes including fruit formation, vegetative growth, stomatal control and tuberization (Wickland and Hanzawa, 2015). This diversity of functions has been the result of gene duplication events, followed by sub- or new-functionalization. The evolution of *FT* proteins has had consequences in the diversification of plants, adaptation and domestication, showing that the molecular plasticity of a single essential gene can direct the evolution of plants and provide key evidence of the mechanisms involved. The duplication of *FT* sometimes results in the presence of many copies of *FT* paralogs in a single species, which may have different spatio-temporal expression patterns, as well as different functions (Pin and Nilsson, 2012). Apparently, some *FT* homologs have acquired the function of suppression of flowering during evolution, antagonizing the function of the *FT* paralogs that induce it (Blackman *et al.*, 2010).

At present, the *FT* gene is recognized as a fundamental integrator in the flowering path in angiosperms that responds to endogenous and environmental stimuli (Wigge, 2011). Different orthologs of *FT* induce flowering in a large number of plants from grasses, legumes, ornamentals to woody perennials.

FT function in perennial species

Perennial species have a life cycle similar to annual plants, such as *Arabidopsis*, with some modifications that allow them to live for multiple years, including a juvenile stage that is measured in years and not days, and several reproductive cycles as long of his life before dying (polyarctic reproduction); in the case of species from temperate zones, a period of dormancy that allows them to survive under unfavorable conditions (Callahan *et al.*, 2016). Although the growth habits between perennial and annual plants are different, just as there is a great diversity of flowers between the species of one group and another, the similarity in the use of *FT* as an

integrator in the flowering process evidences a very conserved among angiosperms (Klintenäs *et al*., 2012).

The function of the *FT* gene, as an integrator of the signaling of flowering routes capable of triggering the transition from the vegetative to the reproductive stage, has been evaluated through genetic engineering strategies in woody stem perennial species that naturally take several years (Table 1).

Table 1. Perennial woody stem species in which the *FT* gene has been described functionally, through over-expression by genetic engineering.

Species	Endogenous *FT*	Exogenous *FT*
Malus domestica Borkh. (apple)	Kotoda *et al*., 2010; Tränkner *et al*.,2010	Yamagishi *et al*., 2011; Wenzel *et al*., 2013; Tränkner *et al*., 2010
Populus deltoides W. Bartram ex Marshall (poplar)	Böhlenius *et al*., 2006; Hsu *et al*., 2006	Böhlenius *et al*., 2006; Zhang *et al*., 2010; Tränkner *et al*., 2010
Poncirus trifoliata (L.) Raf. (hardy orange)	Endo *et al*., 2005	
Pyrus communis L. (pear)		Matsuda *et al*., 2009
*Prunus domestic*a L. (plum)		Srinivasan *et al*., 2012
Eucalyptus grandis W. Hill ex Maiden (eucaliptus)		Klocko *et al*., 2016

In these species, overexpression of *FT* orthologous genes, endogenous or exogenous in nature, have achieved significant reductions of up to less than one year in the juvenile stage. This suggests that the *FT* gene plays an indispensable role in the manipulation of flowering time, by shortening the juvenile phase, and can be used as a tool for research and improvement of woody perennial species (van Nocker and Gardiner, 2014).

Accelerated biotechnological improvement of trees

The knowledge of the molecular mechanisms that control the flowering and the technology to manipulate them by genetic engineering open the possibility of shortening the necessary time for the crosses in programs of improvement of perennial species. The potential of accelerated improvement via biotechnological induction of flowering has been demonstrated in the development of new varieties of apple (*Malus domestica* Borkh.) with greater resistance to diseases such as fire blight, or against fungi such as powdery mildew, and scabies, characteristics introduced in commercial germplasm.

The strategy used in *M. domestica* was to first generate a transgenic cultivar with induced flowering and then pollinate it with wild individuals which had quantitative trait *locus,* (*QTL*) specific disease resistance. The seeds obtained from the crosses were screened with molecular markers and crossed with commercial germplasm, always monitored by molecular diagnoses of the presence of the responsible *locus* for such resistance. In this way it was possible to combine this type of gene in a crop in only three years, when by the traditional method it would have required more than 10 and a much larger seedling population (Flachowsky *et al*., 2011; Le Roux *et al.,* 2012).

However, the genetic engineering strategy used can have important repercussions on the morphology and physiology of the plant. The use of potent constitutive promoters such as the tobacco mosaic virus (35S) causes the continuous formation of flowers that cannot be supported by the seedling and, consequently, the fall of many of the fruits; the dominance of apical growth can also be affected in favor of a greater development of branches and shrub type growth habit (Flachowsky *et al*., 2011, Le Roux *et al.,* 2012). In order to avoid these disadvantages, promoters have been incorporated that allow a temporal control in the expression of the gene, such as the heat inducible *Gmhsp 17.5-E* of soybean. By using this promoter to control the heterologous expression of poplar (*Populus trichocarpa* Torr. & A. Gray ex Hook.) *PtFT1* and *PtFT2* gene in apple seedlings, the induction of flowering was

achieved in six of seven transgenic lines by submitting the seedlings for 60' at 42°C daily for 28 days (Wenzel *et al.,* 2013).

Although the results clearly demonstrated the efficiency of both *FT* genes in inducing flowering, *M. domestica* seedlings subjected to heat treatment showed contrasts in them. The seedlings were in the same stage of development, but the percentage with flowering per line and the number of flowers per plant varied considerably between each transgenic line and between the different transgenic lines. This variation may be due to dissimilarities in the copy number of the transgene and the transgene integration site (position effect) (Wenzel *et al*., 2013).

Another perennial species of woody stem in which the induction of flowering has allowed to accelerate its genetic improvement has been the plum (*Prunus domestica* L.). The plum cultivar HoneySweet, highly resistant to Sharka disease caused by the plum pox virus (PPV) (Scorza *et al.,* 2013) was crossed with California prune seedlings in order to segregate in the latter the gene of resistance. To accelerate sexual crosses, the *FT1* gene of *Populus trichocarpa* (*PtFT*) was over-expressed in plum seedlings (Srinivasan *et al.,* 2012); flowering was obtained in less than a year. The seedlings obtained from these crosses were analyzed using molecular markers for the PPV resistance transgene and the *FT* bloom induction gene, the positive ones were crossed with California-type prune to secrete the resistance to PPV from HoneySweet*.*

The use of the plum flowering induction strategy for *PtFT* over-expression allowed to significantly reduce the time required to segregate the PPV resistance genes (4 to 1 year in each cross) by sexual crossings. Another important advantage of the biotechnological induction of flowering, in addition to the significant reduction of the time necessary in the process of plant genetic improvement, is the possibility of using it in the process and not in the product; that is, to be able to segregate outside, through crossing, the transgene necessary to induce flowering and, thus, not to include it in the product (Callahan *et al.,* 2016).

Examples of stable transformation in apple and plum to over-express the *FT* gene, could be difficult to replicate in other perennial species due to

disadvantages such as low efficiencies of transformation and regeneration (recalcitrant plants), which hinder the functional characterization of genes and biotechnological applications. This is the case of the perennial Kiwi species (*Actinidia eriantha* Benth.) Where it was not possible to perform the functional characterization of the endogenous *FT* gene due to the low number of transgenic lines that were obtained (Varkonyi *et al.,* 2013). To overcome the difficulties inherent in perennial species, modern techniques have been proposed, such as the editing of genes with CRISPR / Cas9 (Fan *et al*., 2015), as well as the use of viral vectors that allow transient transformations bypassing tissue culture (Gleba *et al*., 2004; Yamagishi *et al.,* 2011).

Biosecurity of the induction of flowering

One of the main advantages in biosecurity issues of the flowering induction strategy via manipulation of *FT* genes consists in the possibility of the application of genetic engineering in the process, but not in the product. The reduction to less than a year of flowering in perennial species allows to work with seedlings in the greenhouse, crossing them and evaluating them in controlled and isolated environments to contain the possible flow of pollen.

Given the mobile capacity of the stimulus (*FT*) through the vascular conduits from the leaves to meristems, the strategy of induction of flowering can be applied through the use of grafts (Notaguchi *et al*., 2008), in which the rootstock is the over-expressor of the *FT* gene, which guarantees that the fruits and seeds have no modifications in their genome. When stable transformations are used to accelerate the process of sexual crossings, the gene can be segregated outside of plants (Hoenicka *et al.,* 2014). It is also possible to use transient transformations in distant leaves in order to generate the stimulus that will travel to the meristems and produce flowers and seeds free of genetic modifications.

In relation to the induction of flowering by transient over-expression of the *FT* gene, the use of modified viral vectors has been reported that take advantage of the inherent abilities of the virus to transfer and replicate the genetic material,

particularly in those species in which where it is difficult to use recombinant DNA techniques. One example was the use of the *Apple Latent Spherical Virus* vector (ALSV) to over-express the *FT* gene of *Arabidopsis*, in apple seedlings, inoculated by biobalistics in cotyledons after germination (Yamagishi *et al.,* 2011).

The flexibility and biosecurity offered by this strategy promises to bypass natural barriers that for a long time restricted forest species from breeding by selection. In addition to significantly reducing the time needed to improve forest species by sexual crossings, the induction of flowering will make it possible to take advantage of the genetic diversity available among sexually compatible forest species; additionally, the possibility of using biotechnological strategies in the process and not in the product will avoid the costs and delays of the obligatory procedures in genetically modified organisms for the monitoring of possible risks, in which the total cost for the discovery, development and authorization of a new genetically modified crop may exceed USD $100 million (McDougall, 2011).

Perspectives for the forestry sector

At present, more than 30 000 species of plants are covered by the Convention on International Trade in Wildlife Species (CITES, 2017) against over-exploitation due to international trade, with which the producing countries commit themselves in a priority to guarantee its sustainable management. However, in the case of perennial species, the long juvenile stages hinder their reproduction and improvement. For forest species such as mahogany (*Swietenia macrophylla* King.) and cedar (*Cedrela odorata* L.), considered the basis of the tropical timber industry in Latin America and included in the appendixes of CITES, the biotechnological induction strategies of the flowering are a possibility to accelerate the improvement of varieties with commercial characteristics that promote commercial reforestation and reduce the pressure on natural forests; this contributes to the use of its diversity and at the same time to its conservation.

The export of roundwood worldwide for 2015 amounted to 121 billion m^3 with a value close to $ 15 billion dollars; of this total, the non-coniferous species produced

34 % of the volume and 51 % of the total value (FAO-STAT, 2017). The ability to accelerate the genetic improvement of forest angiosperms through the biotechnological induction of flowering is a disruptive technology that will add value to the forestry sector in a different way, allowing to go beyond the selection improvement that was restricted to forest species due to its long juvenile stages, being able to segregate genes of resistance to biotic and abiotic stress in commercial germplasm.

The flowering induction strategy also has the potential to contribute to the exploitation of the biological diversity of the angiosperm perennial species at a speed never before experienced by the forest sector, with the potential to increase the current production volumes of forest species non-coniferous to meet the needs of timber and energy resources for industrial and domestic uses, increase the flow of foreign currency for export of forest resources, while boosting the economy of rural sectors associated with forest use.

Despite the advances achieved in the functional characterization of the *FT* gene in a wide range of perennial species, in order to take advantage of the biotechnological strategy of induction of flowering in genetic improvement programs of perennial species, it is necessary to advance in the obtaining of genomic information of these plants as well as in the development and validation of fast, economic and efficient techniques that allow us to avoid the difficulties of the perennial species, among them the low efficiencies of transformation and regeneration.

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Conflict of interest

The author declares no conflict of interest.

Contribution by author

Rafael Urrea López: responsible for the design and execution of the described study and for the preparation and correction of the manuscript.

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