Abstract

Despite the huge potential benefits offered by genetically modified (GM) citrus, field releases raise concerns about their potential environmental impact and the possibility to show unexpected deleterious effects from an agronomic view. The main concerns raised by the use of genetic transformation to improve this long-lived crop, of vegetative propagation and complex reproductive biology are: (1) the transfer of transgenes via pollen to compatible varieties of *Citrus* and relatives; (2) the stability of the transgenes in the long-term; (3) the occurrence of adverse pleiotropic effects derived from the integration and expression of the transgenes on the main agronomic and phenotypic crop characteristics. All these issues have been extensively studied in other annual GM crops that are already commercial or non-commercial yet. However, since the use of genetic transformation in improving fruit trees is still in its infancy, currently there is very little information on biosafety and transgene stability for these crops. Therefore, the future of transgenic trees in commercial agriculture remains uncertain, though we already have the technology to produce them. On the other hand, in the case of citrus, there are neither commercial transgenic varieties nor unequivocal evidence that this tool could be really useful to deal successfully with specific improvement goals. Achieving improvement goals so important as improving the nutri-functional quality of citrus fruits through genetic engineering could contribute to a wider acceptance of this technology by the public, since it is an improvement addressed to the consumer first.

In this work, we have faced some of the aspects which are greatly limiting the acceptance and marketing of GM citrus, by (1) conducting a field release experiment with GM citrus to assess their environmental safety and the lack of adverse agronomic effects (2) addressing an objective to improve the nutri-functional quality of orange fruit through metabolic engineering in order to strengthen their healthy properties.

The field experiment consisted of a planting of transgenic citrus trees carrying only the *uidA* and *nptII* marker genes, and its purpose was to study the feasibility of genetic transformation in improving commercially important citrus genotypes. This experimental orchard allowed us to estimate the maximum frequency of transgenic pollen dispersal under conditions of open pollination and to study genetic, phenological and environmental factors that determined it, in order to propose appropriate transgene containment measures for future GM citrus plantings. It also served as a first approach to address basic issues as the study of the stability of transgene expression in the long term (after 7 years of establishment in the field) under real agricultural conditions and its potential impact on the morphology, phenology and fruit quality of transgenic citrus. These studies, though do not solve all concerns regarding GM citrus, provide crucial information about environmental biosafety and behaviour in the field, so far non-existent, which can serve as a basis to design future field trials with GM citrus and to guide case-by-case regulatory policies for new plantings.

Moreover, in this work we have succeeded in developing a strategy to induce early fruit production and increase the content of β-carotene (pro-vitamin A, with high antioxidant capacity) in the pulp of a sweet orange variety by metabolic engineering. This strategy consisted of RNAi-mediated silencing of a β-carotene hydroxylase gene from orange (*CsβCHX*), involved in the conversion of β-carotene into xanthophylls, combined with overexpression of the *FLOWERING LOCUS T* gene from orange (*CsFT*) in juvenile transgenic plants of Pineapple sweet orange. Subsequent tests with the animal model *Caenorhabditis elegans* demonstrated that the enriched orange exerted an *in vivo* antioxidant effect 20% higher than isogenic control oranges. This is the first successful example of metabolic engineering to increase the content of β-carotene (or any phytonutrient) in orange and demonstrates the potential of genetic engineering for nutritional enrichment of woody fruit crops.