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Gene Editing in *Prunus* Spp.: The Challenge of Adapting Regular Gene Transfer Procedures for Precision Breeding

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Abstract

Successfully gene editing (GE) in *Prunus* spp. has been delayed due to its woody nature presenting additional difficulties in both, proper regeneration protocols and designing efficient gene transfer techniques. The availability of adequate, single cell culture techniques for GE such as protoplast regeneration, is a limiting step for the genus and for this reason, the improvement of regular regeneration protocols and finding more efficient techniques for the delivery of the “editing reagents” seem to be a reasonable strategy to incorporate GE in the genus. During the last 10 years, we have focused our efforts optimizing some previous regeneration and gene transfer procedures for Japanese plum (*P. salicina*), sweet cherry (*P. avium*) and peach (*P. persica*) to incorporate them into a GE technology on these species. In parallel, delivery techniques for the CRISPR/Cas9 editing components, i.e., guide RNA (gRNA) and Cas9, have been developed with the aim of improving gene targeting efficiencies. In that line, using DNA virus-based replicons provides a significant improvement, as their replicational release from their carriers enables their enhanced expression. Here, we make a brief overview of the tissue culture and regeneration protocols we have developed for *P. salicina*, *P. avium* and *P. persica*, and then we proceed to describe the use of *Bean yellow dwarf virus* (BeYDV)-derived replicon vectors to express the editing reagents *in vivo* and to evaluate their editing capability on individuals derived from *Agrobacterium*-mediated gene transfer experiments of these species. We show part of our characterization assays using new BeYDV-derived vectors harboring multiple gRNAs, the Cas9 gene, and the green fluorescent protein reporter gene. We also describe a dedicated genome analysis tool, by which gRNA pairs can be designed to address gene deletions of the target genes and to predict off-target sequences. Finally, as an example, we show the general results describing GE of the peach TERMINAL FLOWER 1 gene and some preliminary characterizations of these materials.

Keywords: LSL-based DNA replicons, geminivirus, gene editing, CRISPR/Cas9, *Prunus* genetic transformation

1. Introduction

The Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9 system, discovered as an adaptive line of defense against viral infection in *Archaea* [1], is a common gene editing (GE) technique that allows for the direct generation of targeted sequence modifications in the genome. Its biotechnological use in plants represents a valuable gene expression tool that can contribute enormously to plant breeding. However, this so-called precision breeding, based on these GE techniques and its application in *Prunus* spp., requires developing three fundamental areas: a) tissue culture techniques, b) genome knowledge, and c) molecular biology tools for the delivery of GE components to the cell.

In *Prunus* spp., a huge amount of research has been carried out regarding regeneration and gene transfer protocols using different explants and strategies to improve these procedures [2]. From that research, current gene transfer procedures in the genus include some technical pipelines for whole plant production in species such as *P. salicina*, *P. domestica*, and certain genotypes in *P. avium*. In parallel, increased genome information for the genus is now available, and the advent of genome drafts for *P. persica*, *P. mume*, and *P. avium* have contributed to the feasibility of these new techniques, enabling the prediction and analysis of candidate genes [3]. Finally, improved molecular biology tools, including new expression vectors for GE components in the cell, can now be designed and built in an expedited manner, making the use of these technologies in the genus possible.

In the last decade, we have been immersed in implementing these components to enable precision breeding in the genus. Various tissue culture protocols for the regeneration of peach, plum, and sweet cherry genotypes have been assessed and improved. From that work, regeneration systems for diverse genotypes of these species have been deduced, achieving the first milestone in precision breeding. We also took the available genome information for some of these species and built a genome desktop for their analysis using dedicated bioinformatic components that allowed for the design of the GE key elements, i.e., the guide RNAs (gRNAs), and scored their effect for both on- and off-targets in those genomes. Finally, and due to the highly recalcitrant nature to regenerate found in all of these genotypes, we assumed the generation of new expression vectors for delivery of the GE elements to regular multicellular explants commonly used in our regeneration procedures; from this, traceable virus DNA-replicon derived vectors, with higher expression efficiency in the plant cell and offering the chance of generating either transgenic or non-transgenic edited individuals, have been developed.

2. Tissue culture systems in *Prunus* spp.

A considerable amount of the research carried out regarding *Prunus* spp. regeneration has been based on the use of multiple explant types and culture approaches. To date, most of the *Prunus* genotypes are still recalcitrant to transformation, either because of the lack of regeneration procedures or due to the absence of identified *Agrobacterium* genotypes that are competent for gene transfer.

Since we reviewed this topic 10 years ago [4], only a few examples of successful *Agrobacterium*-mediated transformation have been achieved in this genus and other fruit and nuts species [5]. These successful experiences include plum (*P. domestica*), Japanese apricot (*P. mume*), Japanese plum (*P. salicina*), and apricot (*P. armeniaca*). Whereas a remarkable cultivar/variety-dependence has been observed in these successful experiences, reliable and reproducible systems are yet to be developed for most *Prunus* spp. [2, 5].

Despite these technical constraints, the mentioned technical procedures in some *Prunus* species have resulted in the generation of a portfolio of interesting examples in the arena of genetically engineered trees, with improved traits deserving attention in agriculture. ‘HoneySweet’, one of the main products generated during the previous decade, came from a successful regeneration procedure described in *P. domestica*. ‘HoneySweet’ has been liberalized in the US by the Food and Drug Administration and the Environmental Protection Agency as a new *Plum pox virus*-resistant variety, considering trees safe for both the environment and consumers’ health. In the last decade, derived from the same transformation system in *P. domestica*, the same group developed the “FasTrack” breeding approach, an advanced fruit tree breeding system that uses transgenic European plums that are continually flowering and, in that way, overcome the limitations of juvenility and dormancy processes producing the first generation in one year [6]. Also, in this period, primary tissue culture work in the sweet cherry rootstock ‘Gisela 6’ [7] has led to improved gene transfer and regeneration methodologies for ‘Gisela 7’, allowing for the generation of *Prunus necrotic ringspot virus* (PNRSV) resistant individuals through the use of RNA interference (RNAi) for viral sequences [8]. Interestingly, these materials have led to one of the few studies in woody plant species in which small RNAs have been demonstrated to be transported from rootstock to scion, making ‘Emperor Francis’ scions become resistant to PNRSV [9]. Finally, in 2019, we developed a transgenic Japanese plum tree exploiting the RNAi methodology to target a gene linked to *Plum pox virus* (PPV)-susceptibility, thus generating individuals resistant to PPV [10].

2.1 Regeneration in *Prunus salicina*

Since the first work on *in vitro* propagation described by Rosati *et al.* [11], Japanese plum has been demonstrated to be suitable for *in vitro* regeneration [12] and genetic transformation [13]. Based on the use of hypocotyls isolated from mature embryos, several varieties, such as ‘Early Golden’, ‘Shiro’, ‘Angeleno’ and ‘Larry Ann’, were responsive to culture treatment in the presence of thidiazuron (TDZ) and indole-3-butyric acid (IBA) as supplements of regular Murashige and Skoog (MS) [14] media. These procedures were derived from a previous description for European plum (*P. domestica*) reported by Mante *et al.* [15] and later improved by Padilla *et al.* [16].

In our hands, the use of hypocotyls has allowed for a reproducible gene transfer and regeneration procedure for several Japanese plum varieties (**Figure 1A–D**). Slight variations from the primary protocol described by Urtubia *et al.* [13] have allowed expanding this procedure to the use of epicotyl sections as starting explants, increasing the number of useful material when mature fruits are collected.

Figure 1.

In vitro Japanese plum (*Prunus salicina* cv. Angeleno) regeneration. After infection with *A. tumefaciens* GV3101, hypocotyl explants are subjected to regeneration procedures, achieving whole plant formation between 6 and 8 months of experimentation. A, plum explants (hypocotyls) in co-culture in LP1-medium. B, transformed explants in regeneration medium MSR2 for budding induction. C, shoots transferred to MS03 medium for improving shoot development. D, after elongation, shoots are transferred to WPM rooting medium.

Agrobacterium-mediated transformation of Japanese plum explants requires co-culturing (2–3 days in darkness, usually using strain GV3101) and regeneration on LP-derived medium [17] supplemented with picloram (LP1 medium). For shooting, explants are transferred to MS medium supplemented with variable ratios between TDZ and IBA (MSR2 medium), depending on the variety. Shoot elongation is achieved by cultivation in MS03 medium, i.e., MS-based medium supplemented with benzylaminopurine (BAP). Finally, rooting is achieved by culturing these shoots in a woody plant medium (WPM) [18] supplemented with 1-naphthalene-acetic acid (NAA) and kinetin (WPM rooting medium). Regarding the efficiencies, variable and acceptable values have been obtained depending on the starting explant and the variety assayed, for instance, whereas ‘Larry Ann’ hypocotyl sections reach about 3%, epicotyl segments of the same genotype have shown 17%.

2.2 Regeneration in *Prunus avium*

Very few reports have successfully achieved regeneration, and first procedures used expanded leaves of commercial varieties, including ‘Lapins’, ‘Burlat’, ‘Napoleon’, and ‘Sweetheart’ [19, 20]. These works used WPM supplemented with the growth regulators NAA, BAP, and TDZ. Later, improved regeneration efficiencies were described for ‘Schneiders’, ‘Sweetheart’, ‘Starking Hardy Giant’, ‘Kordia’, and ‘Regina’ using leaf and nodal segments, which were achieved by the combined use of WPM plus Driver and Kuniyuki medium (DKW) [21] or applying Quoirin and Lepoivre medium (QL) [22]. As best growth regulators, these experiments included TDZ and IBA. Using a different approach, mature cotyledons cultured in solid QL medium bound to a 10-day darkness incubation regime led to the genotypes ‘Vista’, ‘Sunburst’, ‘Tehranivee’, ‘Vouge’, and ‘Heidelfingen’ to be regenerated [23]. That shooting capability was lost by some varieties when darkness was eliminated.

Better results have been reported in other commercially important genotypes, including sour cherry (*P. cerasus* L.) [7, 24], black cherry (*P. serotina* Ehrh.) [25], and the cherry rootstocks ‘Rosa’ (*P. subhirtella* autumnno) [26], ‘Gisela 6’ (*P. cerasus* x *P. canescens*) [7], ‘Colt’ (*P. avium* x *P. pseudocerasus*) [27]. In general, these protocols have cultured leaf explants in media, such as QL plus BAP, and WPM plus BAP and IBA, to produce whole plants in a 4–6 month period.

Based on those previous descriptions and considering our results in *P. salicina* regeneration, we developed a consistent procedure for sweet cherry varieties, such as ‘Bing’, ‘Van’, and ‘Lapins’. The system is based on the use of epicotyl segments, which are first co-cultured with *Agrobacterium* (mostly strain GV3101) in MP2 medium (LP-derived medium supplemented with picloram) in darkness for 2–3 days. Subsequently, explants are transferred to B15-medium for budding induction; B15 medium is also based on LP micro- and macro-nutrients, supplemented with the growth regulators TDZ and 6- γ,γ -dimethylallylaminopurine (2iP). Shooting elongation is then achieved in D03 medium (DKW-based medium supplemented with BAP and IBA), first culturing the explants in Petri dishes and then using jars. Finally, elongated shoots are rooted in WPM medium (same as in plum regeneration) (**Figure 2A–D**). Observed efficiencies in this system reach about 4% (4.33% and 3.75% for ‘Lapins’ and ‘Bing’, respectively). Hypocotyls have also proven to be responsive to these procedures, although efficiencies are considerably lower.

2.3 Regeneration in *Prunus persica*

Peach highlights as one of the more recalcitrant species regarding the *in vitro* regeneration process. Although the first descriptions for *in vitro* propagation were

Figure 2.
In vitro sweet cherry (*Prunus avium* cv. *Lapins*) regeneration. After infection with *A. tumefaciens* GV3101, epicotyl explants are conducted to the regeneration procedures achieving whole plant formation between 6 and 8 months of experimentation. A, transformed explants in regeneration medium B15 for budding induction. B and C, early shootings transferred to D03 medium for elongation and development, first in Petri dishes and then in jars. D, elongated sweet cherry plantlets are transferred to WPM rooting medium.

described as early as 1982 [28], all the considerable research published to date pursuing the development of a reproducible regeneration system based on adult tissue as starting explants has shown this to be more complex than originally thought [2].

Considering those experiences and our own experimentation, we have been focused on improving efficiencies for the use of immature seeds as a source for explants [4], which in our hands represents the only reproducible procedure to achieve peach regeneration with a reasonable time frame. Since then, improvements on this pipeline have contributed to the generation of new individuals by an adequate protocol for the application of genetic engineering.

The protocol for peach regeneration using seed explants was formerly derived from descriptions found in the work by Gentile *et al.* [17], and it was initially applied on ‘O’Henry’ and ‘Rich Lady’ explants. One key point resulted in the use of immature cotyledons 70–90 days after bloom, which are cultured in LP modified medium. In general terms, after transformation and co-culture with *A. tumefaciens* strain GV3101, peach cotyledons are transferred to solid LP medium supplemented with BAP and NAA for callusing. Induced calli, usually observed after 3 to 6 months, are transferred to 03BAP-LP medium (LP medium supplemented with BAP and IBA) for budding induction. Shoot elongation is then achieved by transferring the shoots to CP2 medium (LP-derived medium supplemented with growth increased ratios of BAP/IBA). Finally, peach shoots are transferred to 03BAP-MP medium (LP medium using MS micronutrients plus growth regulators BAP and IBA) for elongation and rooting (**Figure 3A–D**). We have observed that embryo-derived explants from these immature fruits are also responsive to these treatments, and after infection, co-culture, and callusing in LP medium, explants must be cultured in 03BAP-MP medium.

Figure 3.
In vitro peach (*Prunus persica* cv. *Rich Lady*) regeneration. After infection with *A. tumefaciens* GV3101, cotyledons are conducted to the regeneration procedures achieving whole plant formation after 8–12 months of experimentation. A, transformed explants are cultured in LP-derived medium for calli induction. B, Calli are then transferred to 03BAP-LP medium for budding. C, shoots are transferred to CP2 medium for elongation. D, shoots are transferred to 03BAP-MP medium achieving further elongation and rooting.

For rooting, plantlets regenerated from either cotyledon or embryo explants may need to be placed for 1 month in M1 medium (MS-derived medium supplemented with NAA); after this period, plantlets can be cultured back to 03BAP-MP medium. The procedure to achieve complete *in vitro* rooted plants can be as short as 8 months. However, longer times can be needed. In terms of regeneration efficiency, around 2% of embryo explants from ‘Elegant Lady’ can regenerate into plants. The regeneration rate for cotyledons in ‘Elegant Lady’ is in the region of 0.5–1%.

3. Genomes and CRISPR/Cas9 technology

3.1 Available genomes in *Prunus* and a CRISPR/Cas9 analysis tool for them

Prunus spp. belong to the *Rosaceae* family are among the most relevant groups in terms of genome drafts already available. The Genome Database for *Rosaceae* (GDR, <https://www.rosaceae.org>) provides public access to genomics, genetics, and breeding data for the family [29].

Peach was one of the first sequenced species in this group [30], and a reviewed version of the database (Peach v2.0) was recently made available [31]. The generated information was obtained from the doubled haploid cultivar ‘Lovell’, showing a relatively small genome size [265 Mb; diploid $2n = 16$]. In addition to its short juvenile period (2–3 years) and self-pollination capacity, this condition makes this species a useful model for the *Rosaceae* family [32].

Genome assemblies for sweet cherry have been generated since 2017 for ‘Satonishiki’ [33], ‘Karina’ [34], and ‘Big Star*’ [35]. These works showed a slightly bigger genome size [353 Mb; diploid $2n = 16$] compared to peach, although with a greater synteny between both genomes.

Just recently, a chromosome-level assembly for Japanese plum (*P. salicina*) has been described [36], revealing an intermediate genome size [284 Mb; diploid $2n = 16$] relative to the other two species. Phylogenetic analysis showed *P. salicina* having a close relationship with the other two already sequenced species, *P. mume* [37] and *P. armeniaca* [38].

The availability of these genome drafts in *Prunus* enables advancement toward dedicated bioinformatics tools to carry out faster and safer analyses regarding the GE technology.

Targeted mutagenesis by CRISPR/Cas9 involves making guide RNAs (gRNAs) that target customized sequences in the genome of a cell to direct the Cas9 nuclease activity to generate double-strand DNA breaks close to the gRNA-joining location. Experimentally, this gRNA is a short synthetic RNA composed of a) a scaffold sequence that gives a secondary structure that is necessary for Cas9-recognition and assembly and b) a user-defined 20-nucleotide sequence (20-nt) spacer that determines the genomic target to be modified. Consequently, the target sequence recognized by the spacer will be a protospacer sequence contained in the target genome, located right next to a motif recognized by Cas9 for DNA cleavage, which is an NGG nucleotide arrangement called protospacer adjacent motif (PAM). Given its impact, the advent of CRISPR/Cas9 technology has led to the discovery and characterization of new nucleases recognizing different PAM motifs and even to Cas9 genetic engineering to develop modified enzyme activities [39].

Whereas the RNA scaffold sequence in the gRNA is operatively a regular, non-changing component in the system, the 20-nt spacer will be the key molecule to deduce from any available genome draft. As shown in **Figure 4**, from the genome information, it is possible to acquire the number of NGG motifs for each species, and from these data to consider the 20-nt adjacent to each one of these

Figure 4. Target sequences for the gRNA + Cas9 editing modules in some fruit tree crop species. Number of NGG motifs found in the sweet cherry and peach genome drafts from which data considering the 20-nt adjacent sequences are considered as all the putative CRISPR [gRNA + Cas9] recognition sites in each genome. Modules with unknown ("N") sequences are excluded from the CRISPR search database (see www.fruit/tree/genomics.com; "Biotools" bar). tcrRNA, trans CRISPR RNA.

motifs, creating a database for all the putative gRNA + Cas9 recognition site in each genome. This database will represent all the recognition sites for the CRISPR/Cas9 system in each genome and will allow us to predict both the on-target as the possible off-target sequences. As known for most of the genome information, several drafts still need to be confirmed due to sequencing errors or uncertainty and that information is filled with unknown nucleotides ("N"), information that will be removed from its corresponding protospacer databases.

Due to the probability of having nucleotide mismatches at the time of recognition between the gRNA and the protospacer, we can expand the 20-nt + PAM databases to consider the occurrence of mismatches, starting from one mismatch to as many pairing errors as we want to analyze. These expanded databases will increase the number of candidate off-targets in a particular genome, improving the predictive power of our *ex-ante* analysis for the right gRNA selection.

3.2 Vectors for gene editing

Despite its novelty, the delivery to the cell of CRISPR/Cas9 relies on regular and efficient gene transfer technology. The status of gene transfer procedures in tree fruit crops has been recently reviewed, including GE [5]. That work stressed the additional problems in using GE technologies in these species, considering their clonally propagated nature. In fact, the use of stable transformation in these species for gRNA + Cas9 delivery into the cell (explants) is not suitable because, unlike annual crops for which the transgenes can eventually be bred out after the mission of GE is achieved, breeding out these transgene sequences is a difficult, labor-intensive, and frequently time extensive process. Also, the use of crosses could lead to eliminating the identity and valuable traits of the variety.

For these reasons, efforts to improve the delivery of the editing components need to be considered in the case of fruit tree crops. One of the more convenient procedures to overcome these inconveniences has been the use of non-transgene-involved GE by using a gRNA-Cas9 ribonucleoprotein complex coupled to

protoplasts gene transfer and regeneration. This has been established as proof of concept in apple and grape cells with no final plant generation [40]. In this regard, massive use of this approach will depend on developing plant regeneration procedures from protoplast cells, which in tree fruit crops is quite limited [5].

Improvements in the delivery of GE tools have already been achieved by autonomously replicating viral vectors [41]. The single-stranded DNA (ssDNA) replicons from geminiviruses having been particularly useful. This group represents a large family of plant viruses with small (2.5–5.5 kb) genomes that replicate by rolling circle replication (RCR) in the plant cell nucleus. This process occurs through a double-stranded replication intermediate that also serves as a template for transcribing the viral open reading frames [42]. *Bean yellow dwarf virus* (BeYDV), the smallest member in the family, has been extensively explored as a molecular tool to improve gene expression by generating disarmed versions referred to as “LSL” (LIR-SIR-LIR) [43] or “deconstructed virus” vectors [44]. The three main elements from the virus replication machinery are retained in the LSL vectors, allowing virus replication by RCR to be emulated, thus enabling the transcriptional activation of the included expression cassettes. Two of these elements act in *cis*, the long and short intergenic regions (LIR and SIR, respectively) [45]. The SIR is the origin of replication for minus-strand synthesis and contains transcription termination signals. The LIR contains bidirectional promoter elements and provides a stem-loop structure, which is essential for initiating the RCR of the plus-strand [46]. The third element corresponds to the virus replication initiator protein (Rep/RepA), which acts in *trans* [47] and, therefore, must either be expressed by the viral replicon itself or be externally provided [43, 46].

In gene transfer experiments, the LSL vectors are activated by Rep’s nicking function acting on the LIR components arranged in tandem in the delivered plasmid, which results in the replicative release of the recombinant viral DNA cloned between them [43]. The released DNA then replicates episomally in the nucleus, leading to the efficient expression of the encoded genes [48]. The shuttle capability of LSL vectors was demonstrated when T-DNAs released BeYDV-derived replicons in *Nicotiana benthamiana* cells, allowing for the expression of exogenes, including the green fluorescent protein (GFP) and human vaccine antigens for human papillomavirus and human immunodeficiency virus [49]. More recently, BeYDV-derived replicons have been used in T-DNAs in tobacco [41], tomato [47], and potato [50] to efficiently deliver plant genome editing machineries, such as zinc-finger nucleases, transcription activator-like effector nucleases, and CRISPR/Cas9.

3.3 Building a universal LSL-based vector for gene editing in plants

Considering our weakness in establishing protoplast systems in tree fruit crops, and particularly in *Prunus*, we decided to expand the geminivirus technology by designing and building general vectors that allow for efficient gene transfer experiments in these species that could lead to eventually edited individuals.

Formerly, we built a universal version of a BeYDV-derived LSL vector by assembling all the fragments containing the important components of a DNA replicon, as shown in (Figure 5, “*Agrobacterium* T-DNA + LSL components”). In this vector, named pGMV-Universal (pGMV-U), we incorporated all the elements required for both viral RCR replication and CRISPR/Cas9 gene editing. These include pGMV-U components arranged into multiple gRNA expression cassettes, allowing the individual expression of up to four gRNAs, the sequence comprising a SIR element, the coding sequence for Rep/RepA, and the LIR sequences adjacent to the right and left T-DNA borders, respectively. Finally, the vector included a Cas9 expression cassette (Figure 5, “pGMV-Universal vector”). Overall, a 15,657 bp vector for plant gene-transfer experiments was made (Addgene #112797).

Figure 5. Geminivirus-based vectors for plant gene editing. The genomic organization of geminiviruses is shown on top, including the circular and linearized versions (adapted from [51]). MP; movement protein, CP; coat protein, LIR and SIR; long and short intergenic regions; Rep/RepA, replication initiator protein. T-DNA-derived gene transfer vectors include key regulatory elements from these DNA viruses (Agrobacterium T-DNA + LSL components), a type of vector allowing for an important cloning capacity (cargo). Our first geminivirus-based vector (pGMV-Universal), based on BeYDV genome elements, consisted of the addition of multiple gRNA scaffolds that allow the expression of up to 4 different guide RNAs (gRNAs (4)) and an additional cloning site (Att 1/2). The second-generation (pGEF-Universal) contains the green fluorescent protein expression cassette (GFP).

In a second instance, considering that selection in the gene transfer process is a very limiting decision step in tree fruit regeneration processes, we improved pGMV-U into a traceable version of it by adding a GFP expression cassette (**Figure 5**, “pGEF-Universal LSL vector”). This vector has been named pGEF-U and retains all the previous functions for a geminivirus-based GE vector in plants.

4. Gene editing systems applied to regeneration protocols in Japanese plum, sweet cherry and peach

The regeneration protocols previously established for Japanese plum, sweet cherry, and peach allowed for the *Agrobacterium*-mediated delivery of the editing components through the pGMV-U and pGEF-U vectors.

The ability to replicate LSL vectors in the *Prunus* explants, a non-natural host for BeYDV, was first evaluated using an empty (i.e., with no gRNAs) version of pGEF-U, carrying out different time course analyses after gene transfer.

In *P. salicina*, our preliminary assays of transformation with *Agrobacterium tumefaciens* strain GV3101 carrying pGEF-U vector showed that both ‘Larry Ann’ and ‘Angeleno’ varieties responded well to infection. According to GFP expression detected 7 days post-infection (dpi), we observed that on all evaluated occasions, explants derived from both hypocotyls and epicotyls are infected at a rate greater than 90% for both varieties evaluated (**Figure 6A and B**).

Figure 6. Green fluorescent protein expression during gene transfer in *Prunus salicina* using the pGEF-U editing vector. Japanese plum explants were transformed with *Agrobacterium tumefaciens* strain GV3101 harboring the geminivirus-based vector, and GFP expression analyzed under epifluorescence microscopy at 7 days post-infection. A, *P. salicina* cv. Larry Ann; B, *P. salicina* cv. Angeleno. FITC, fluorescein isothiocyanate filter; GFP, green fluorescent protein filter.

In the same way, our preliminary assays of transformation with *Agrobacterium tumefaciens* strain GV3101 carrying pGEF-U vector evaluated in *P. avium* showed that both 'Lapins' and 'Bing' varieties also responded well to infection. According to GFP expression detected 7 dpi, we observed that explants derived from both hypocotyls and epicotyls are infected at a rate greater than 90% for both varieties evaluated (**Figure 7A and B**).

Evaluations of transformation with *Agrobacterium tumefaciens* strain GV3101 carrying pGEF-U vector carried out in *P. persica* showed that the three varieties assayed, 'Rich Lady', 'Elegant Lady', and 'Red Top', responded very well to infection,

Figure 7. Green fluorescent protein expression during gene transfer in *Prunus avium* using the pGEF-U editing vector. Sweet cherry epicotyls and hypocotyls are subjected to gene transfer experiments with *Agrobacterium tumefaciens* strain GV3101 harboring the geminivirus-based vector, and GFP expression analyzed under epifluorescence microscopy at 7 days post-infection. A, *P. avium* cv. Lapins. B, *P. avium* cv. Bing. FITC, fluorescein isothiocyanate filter; GFP, green fluorescent protein filter.

when we refer to explants derived from embryos, presenting an infection efficiency greater than 85% in all cases. The opposite occurs when cotyledon-derived explants are used, where we observed that, in general, the quantity and quality of the infection are quite variable. Whereas we have been able to obtain infections that reached 90% effectiveness (evaluated by GFP emission), these high rates in the gene transfer will depend on the proper physiological state when explants are collected, which takes place during a limited window of time after bloom (**Figure 8A–C**).

Tracking the GFP expression in these experiments allowed for analyses of pGEF-U at longer post-infection times. In that way, the interaction between explant cells and the vector beyond a stage of transient expression was observed over the regeneration protocols for these species. The longest GFP expression was observed in *P. salicina*, where it was detected even 42 dpi; nevertheless, this condition was observed in just a small number of the initial explants (**Figure 9**). In *P. avium*, the duration of GFP expression has a relatively shorter permanence (28 dpi) compared to Japanese plum and over a much shorter time in the tests carried out in *P. persica*, where GFP emission is detected only until day 21.

These results strongly suggested that our BeYDV-based vectors can successfully infect various types of *Prunus* spp. derived explants, and that they can generate multiple copies of DNA replicons that lead to the expression of GFP and the CRISPR/Cas9 system. Moreover, the screening of GFP expression during the proper time frame enables selecting the explants that bear the highest rates of infection, and consequently, are the most likely to generate successfully edited individuals for specific targets. In the coming sections, we will go over some preliminary results of our GE assays in *Prunus* spp. and the rationale behind our gene selection and experimental design.

4.1 An example: editing of the TERMINAL FLOWER 1 gene in peach

As perennial trees, *Prunus* spp. are grown in temperate climates. In the early fall season, the decrease in temperature and daylight hours promotes entry to the dormancy state, an evolutionary phenomenon relevant for survival during the adverse conditions in winter. In dormancy, certain requirements must be completed to resume growth. The first requirement corresponds to cold accumulation during winter (endo-dormancy) and then heat accumulation at the beginning of spring (eco-dormancy). After an adequate cold accumulation, flowering takes place.

Figure 8. Green fluorescent protein expression during gene transfer in *Prunus persica* using the pGEF-U editing vector. Immature peach embryos and cotyledons are subjected to gene transfer experiments with *agrobacterium tumefaciens* strain GV3101 harboring the geminivirus-based vector, and GFP expression analyzed under epifluorescence microscopy at 7 days post-infection. A, *P. persica* cv. Rich lady. B, *P. persica* cv. Elegant lady. C, *P. persica* cv. Red top. FITC, fluorescein isothiocyanate filter; GFP, green fluorescent protein filter.

Figure 9. Time-course analysis of the green fluorescent protein fluorescence from pGEF-U vector in *Prunus salicina* cv. Larry Ann transformed with *Agrobacterium tumefaciens* strain GV3101. FITC, fluorescein isothiocyanate filter; GFP, green fluorescent protein filter.

Plants continuously sense the environment (i.e., day and night conditions; seasonal cycles) to adapt their metabolism, growth, and development to these diverse conditions. These responses involve changes in gene expression programs to overcome a particular scenario until new conditions demand new responses. Diverse gene programs have been associated with dormancy and flowering signals and include cell cycle regulation, light perception, hormonal signaling, and stress response [52–54]. Two important genes participating at some level in these events are *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER 1* (*TFL1*). Despite *FT* and *TFL1* share a high (~60%) amino acid sequence identity, they function in an opposite manner [55]. Whereas *FT* promotes the transition to reproductive development and flowering, *TFL1* represses this transition [56]. As already mentioned, transgenic European plum over-expressing the poplar *FT1* gene has led to altered dormancy requirement and continuous flowering, enabling what has been named “FasTrack” breeding technology [6]. On the other hand, the flowering repressive function of *TFL1* has been reported in many species of *Rosaceae*, including *Prunus* spp. [57–60]. In pear, silencing of *PcTFL1* and *PcTFL2* through RNAi resulted in individuals that started flowering as early as 4–6 months, with no adverse phenotypic effects [61]. Consequently, *TFL1* is an appealing candidate for producing fast-breeding trees through loss-of-function experiments in stone fruits using CRISPR/Cas9 edition.

4.1.1 Design and strategy

The *TFL1* locus in *Prunus persica* comprises four exons and three introns and spans through 1.3 kb. We chose to simultaneously cleave two different located in exons 2 and 3, removing approximately half of the gene from the genome as a result (**Figure 10**).

Figure 10.
*CRISPR/Cas9 gene editing of the *P. persica* TFL1 gene. A, genomic structure of the locus (PRUPE_7G112600). Depicted are the UTR regions (black segments in the left and right ends), the translation starting site (arrow above the first orange rectangle), the exons (orange rectangles), introns (black segments between exons), and the translation stop site (stop). The approximate locations of the two gRNAs used to generate a 0.58 kb deletion (gRNA1, gRNA2) and the primers employed for deletion screening (S1 and A1) are also indicated. B, peach cotyledons were transformed with pGEF-TFL1 using agrobacterium tumefaciens strain AGL1; representative pictures of infected (GFP+) and uninfected (GFP-) embryo explants under an epifluorescence microscope 8 days post-infection. C, 1.5% agarose gel of PCR products from genomic DNAs derived from embryo explants transformed with pGEF-TFL1, 28 days post-infection. MW: Molecular weight ladder. GFP+: Embryo explants positive for GFP expression. WT: Untransformed, wild-type embryos. GFP-: Embryo explants negative for GFP expression. H2O: no DNA control. Amplicons corresponding to the expected size of the edited products are signaled with white arrows. D, sequencing analysis of amplicons derived from embryo explants infected with pGEF-TFL1. E, acclimatized individuals whose genotypic and phenotypic characteristics are under study.*

Guide gRNAs were selected using the CRISPR-Search tool (freely available at <https://www.fruit-tree-genomics.com/>). This tool, generated by our group, searches for the best pair combinations of gRNAs to induce the deletion of a defined target area within a genomic sequence. As mentioned, one of the main characteristics of our pGMV-U and pGEF-U vectors is their cargo capability for up to four different gRNAs. Thus, both chosen gRNAs targeting *TFL1* were cloned into a single vector, named pGEF-TFL1. The “loss of a large gene fragment” approach has two distinct advantages: first, it allows us to maximize the chances of successfully inactivating our target genes by avoiding silent mutations, and second, it enables the screening of deletions through a straightforward PCR assay (**Figure 10A** and **C**).

We show results from the molecular analysis of embryo explants from *Prunus persica* transformed with the geminivirus-based GFP-Cas9 double gRNA vector pGEF-TFL1 (**Figure 10B**). Twenty-eight days post-infection, infected and uninfected embryo explants were processed for genomic DNA purification. DNA samples were analyzed through PCR, using primers located upstream from gRNA1 and downstream from gRNA2 (**Figure 10A**). As a result, we found an array of unedited and edited amplicons: the bigger PCR products, with a size around 950 bp, corresponding to the unedited form of the *PpTFL1* gene (**Figure 10C**). The second group, comprised of shorter amplicons with varying sizes close to 380 bp, originated from the deletion of the DNA segment between the two gRNAs. The cloning and sequencing of these amplicons confirmed the successful edition of *PpTFL1* (**Figure 10D**). These explants have already regenerated into several new individuals (**Figure 10E**) whose genotypic and phenotypic characteristics remain to be assessed.

5. Conclusions

The traditional breeding strategies mainly rely on natural genetic or induced variability for the incorporation of desired traits into crops. Individuals with interesting traits are selected as parental, and through controlled crosses, siblings with a combined genetic pool are obtained. Intense breeding programs based on the use of a narrow number of parentals have resulted in a considerable loss of genetic diversity. Also, old and newly released cultivars sometimes do not adapt well to the distinct environments in a demanding context characterized by global climate change.

We live today in a fantastic time of massive and increasing knowledge. In the last decade, an enormous amount of information has been made available from massive sequencing technologies in plants. This progress, fused to the biotechnological approaches based on RNAi and, more recently, CRISPR/Cas9 GE, has opened new alternatives in breeding and represent additional and available tools. Due to its commercial relevance and biological properties, the *Prunus* genus is part of these developments.

Today, we have genome drafts for diverse species in *Prunus*, which has opened an enormous opportunity to apply new breeding techniques in the genus. Ten years ago, the European and Japanese plums were successful examples in the genus, and these advances allowed for the current knowledge and possible expansion of tissue culture techniques to other species. As we show here, the need for consistent technical procedures in *Prunus* regeneration is a starting point for applying this “precision breeding” approach. It is also relevant to consider that few successful steps forward in the area are indeed huge advances. For instance, the generation of genetically engineered individuals – beyond a “proof of concept” – can also be considered potential parentals for breeding. This is also relevant when rootstock technology, based on these developments, can be projected.

The complementarity between RNAi and GE seems evident today. Whereas candidate gene functions have been clarified by the first, efforts in generating non-transgenic gene-edited individuals results are encouraged by those findings. In this regard, the strength of counting with reviewed genome drafts in these species is a requisite. It will be interesting to see how, in the future, the ever-increasing amount of genetic information will allow us to identify new targets for GE and perhaps “export” specific traits of interest from one cultivar to another in a straightforward manner, greatly speeding up the traditional breeding process.

As shown, our weaknesses in tissue culture systems have been overcome by improving the delivery procedures of the editing tools. We have built LSL-derived vectors to increase our relatively low regeneration efficiencies, opening a possibility of using regular multicellular explants in the generation of eventual edited non-transgenic individuals. Also, tracking of the processes during tissue culture has resulted in vital relevance, allowing us to prioritize explants in which the editing process is taking place.

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