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Genetic Transformation Strategies in Fruit Crops

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1, Introduction

Genetic transformation provides the means for adding single horticultural traits in existing cultivars without modify their commercial characteristics. This capability is particularly valuable for perennial plants and fruit tree species, in which conventional breeding is hampered by their long generation time and juvenile periods, complex reproductive biology, high levels of heterozygosity, limited genetic sources and linkage drag of undesirable traits from wild relatives. In addition, gene transfer technologies for fruit tree species take the inherent advantage of vegetative propagation used for their reproduction, which allowed for the application of a high scale production of the desired transgenic line starting from one successful transformed line. Despite this opportunity, final setting of transformation protocols in this type of species, endures major limiting factors preventing the development of new varieties: a) explants recalcitrance to regenerate adventitious transformed shoots and b) a limited regeneration capability, usually extended to just few genotypes (i.e. cultivar dependence).

This chapter illustrates the road between the establishment of transformation methodologies on particular species of *Vitis* spp. and *Prunus* spp. and their use as technical baselines for achievement of transformation procedures in new, eventually more recalcitrant, cultivars or genus members.

1.1 Genetic transformation of fruits in the current research era

Genetic improvement of fruit trees is essential for increasing fruit production. For most of these species, the desired new varieties contemplate the presence of agronomic and horticultural traits related to propagation, yield, appearance, quality, disease and pest control, abiotic stress and shelf-life. Incorporation of these traits into the genetic backgrounds of species by conventional breeding needs overcome some major disadvantages, including long juvenile periods and reduced possibility of introgression of the suitable traits (when available) into commercially relevant cultivars. Although currently the use of new technologies based on high throughput platforms for sequencing and genotyping has deeply contributed to accelerate the association of molecular markers and major genes to these relevant traits, there exists a bottle neck in this strategy when

phenotyping must be carried out. In addition, breeding by controlled crosses is hampered due to factors specifically related to complex characteristics belonging to these species, such as delayed flowering, unsuccessful fruit setting due to abortive embryos, massive fruit drop, and self-incompatibility barriers found in many of them.

Genetic transformation represents inherent advantages for fruit tree improvement, although in fruit trees this area of research is not a routine procedure. The transversal negative perception about the "transgenic technology" is added to an additional degree of difficulty for setting up adequate technical systems in fruit tree species. Eventually, if a proper regenerative system has been established, any DNA construct designed for either a major gene over-expression or gene silencing (i.e. interfering RNA´s *in vivo* generation) can be introduced into a desired genome. Consequently, the feasibility of genetic modification relies on adequate technical systems which allowed for results in a reasonable time frame. Regardless the final objectives of transformed events (a product or fundamental research), highly regenerative systems for explants production and whole plants regeneration are key steps of fruit tree genetic transformation. In addition, the relevance of these procedures is even higher when an era of candidate genes evaluation has begun as a result of the current knowledge about genomes.

2. Use of grapevine systems as a model in fruit species

2.1 Grapevine somatic embryogenesis and genetic transformation of somatic embryos

Since its first report in 1976 (Mullins and Srinivasan, 1976), somatic embryogenesis (SE) in *Vitis vinifera* L. has been described in different cultivars and their hybrids (Martinelli and Gribaudo, 2001, 2009), becoming the most efficient procedure for the generation of *in vitro* cultures prone to genetic transformation (Stamp and Meredith, 1988; Scorza *et al.*, 1996; Martinelli *et al.*, 2001a; Iocco *et al.*, 2001; Torregrosa *et al.*, 2002; Hinrichsen *et al.*, 2005, Li *et al.*, 2008). As described by Ammirato (1983), SE is understood as the initiation of embryos from plant somatic tissues closely resembling their zygotic counterparts. As a fruit species, grapevine SE is not a routine procedure that can be easily and efficiently reproduced among different cultivars (Martinelli *et al.*, 2001; Araya *et al.*, 2008). Grapevine SE has been successfully reached using as source explants stamens and pistils (Rajasekaran and Mullins, 1983; Martinelli *et al.*, 2001a, Araya *et al.*, 2008), unfertilized ovules (Mullins and Srinivasan, 1976), ovaries (Martinelli *et al.*, 2001), leaves (Martinelli *et al.*, 1993), petioles (Martinelli *et al.*, 1993), and tendrils (Salunkhe *et al.*, 1997). In the conventional approach, grapevine SE is induced to the generation of pro-embryogenic (PE) and embryogenic (E) cell masses by cultivation of these explants in solid X6 medium using TC agar Petri dishes for 30 days (Li *et al.*, 2001). X6 corresponds to a modified MS (Murashige and Skoog, 1962) medium lacking glycine and supplemented with KNO_3 and NH_4Cl as the sole nitrogen source, in addition to sucrose, myo-inositol, and activated charcoal. For transformation, cells are pre-conditioned by a seven days treatment in DM (Driver and Kuniyuki, 1984) solid medium and then infected with *Agrobacterium tumefaciens* by immersion of explants in liquid DM medium containing the bacteria. After two days in co-cultivation, an early selection is applied using solid DMcck (DM medium supplemented with carbenicillin, cefotaxime, and kanamycin) medium for 21 days. Transformed cells are, again, induced to generate E cells in solid X6

medium up to appearance of mature structures and fully developed somatic embryos, which are picked up and harvested as putative transgenic lines (Figure 1).

SE process based on the use of Petri dishes is illustrated for both leaves and inflorescences as source explants. This process leads to somatic embryo generation (embryogenic masses) prone for *Agrobacterium* infection. After co-cultivation, a selection step is applied by 21 days. Afterwards, embryo development leading to the regeneration of whole plants, acclimatization and field trial of the obtained individuals are carried out. The field trial shown in Figure 1 corresponds to an assay of genetically modified plants generated to introduce tolerance to the fungus *Botrytis cinerea* carried out since 2004 at La Platina Station in the National Institute of Agriculture, Santiago, Chile.
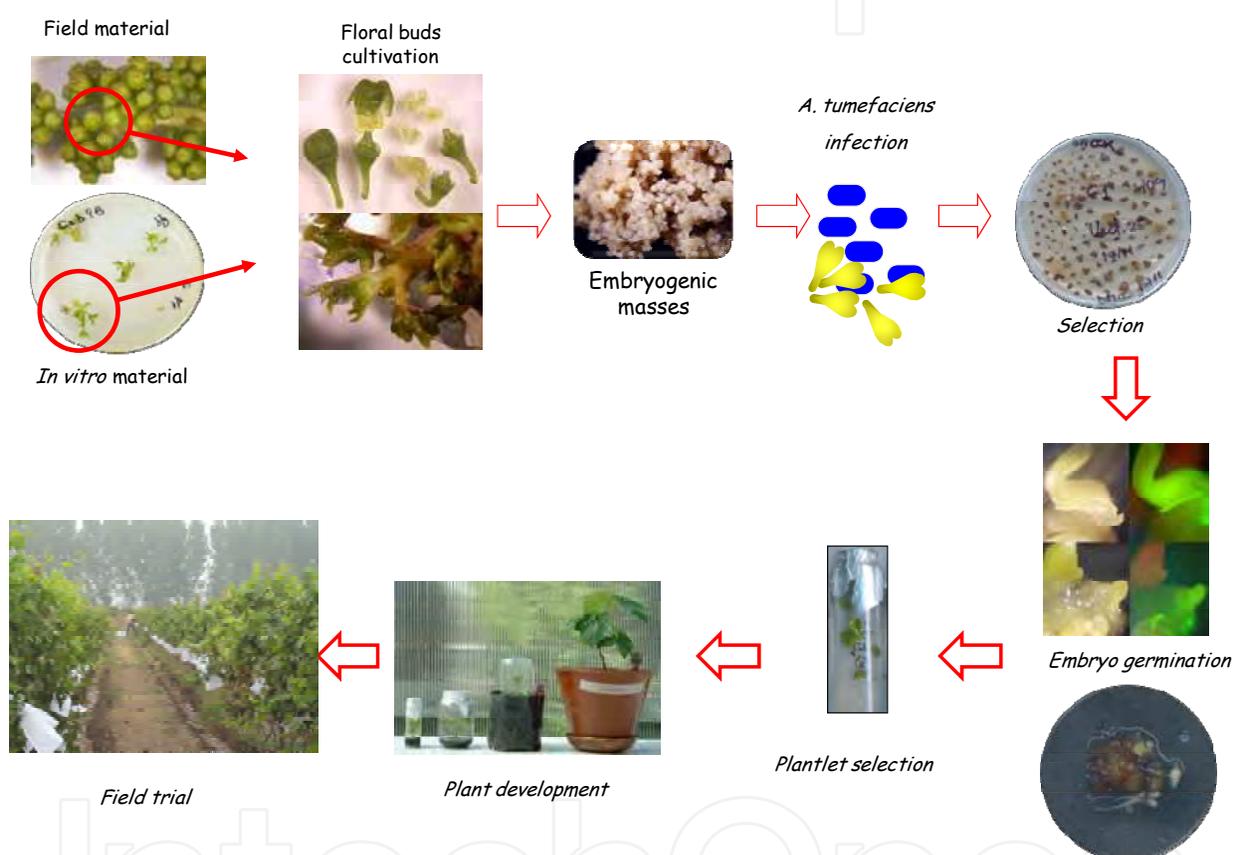

Fig. 1. Conventional work flow involved in somatic embryogenesis (SE) and *Agrobacterium*-mediated genetic transformation of grapevine ´Thompson Seedless´.

2.2 Current requirements in grapevine genetic transformation

The convergence of genome sequencing studies on *V. vinifera* cv. ´Pinot Noir´ (Jaillon *et al.*, 2007; Velasco *et al.*, 2007) and a high through-put transformation pipeline to carry out the evaluation of candidate genes, seem a current major priority. The SE-mediated transformation process of grapevine has not been directed to the massive generation of transformable explants. Improvements to the technology have recently showed up by maintenance of embryogenic cultures using suspension cultures in flasks. These efforts did not report major morphological or anatomical differences in the generated E and PE masses when compared to the above described procedures using solid media (Jayasankar *et al.*,

2003). Recently, Li *et al.* (2008) improved this fundamental methodology by introduction of a flask-based calli processing before starting the second round of sub-culturing in solid X6 medium. This additional step consisted of four consecutive sub-cultures using agitated flasks containing DMcck; i.e. under a permanent selection by increasing the kanamycin working concentrations (in grapevines this is referred as concentrations from 20 to 50 mg/L). A final selective pressure is applied (for instance 100 mg/L) by addition of kanamycin to solid DMcck Petri dishes. The processed calli were reinserted into the second round of culturing in solid X6 medium dishes, until embryo harvesting and development for evaluation.

In general terms, the whole process requires a total time of 24 to 25 weeks and leads to the generation of candidate genetically modified plantlets ready for a primary, PCR-based, screening.

Regardless the strategy (solid media Petri dishes or solid media plus the inclusion of a flasks' step), both routes share a critical and consistent problem referred to the massive generation of adequate amounts of E or PE masses supporting routine transformation experiments.

2.3 Optimizing somatic embryogenesis platforms. Different strategies

It is accepted that the developmental stage of source explants is of great importance in grapevine SE setting (Martinelli and Gribaudo, 2009). Commonly, SE systems in grapes are initiated from stamens and pistils and responses are variety-dependent. The best developmental stages to initiate embryogenic cultures have been deduced for some genotypes using the basis of phenological stages of inflorescences (Dhekney *et al.*, 2009); whereas stamens and pistils from some cultivars such as ´Pinot Gris´ must be collected at early developmental stages; other genotypes such as ´Merlot´, ´Sauvignon Blanc´ and ´Freedom´ must be induced using explants at more advanced maturity stages.

In vitro leaves have been also proposed as source explants for SE induction in grapevines. Although lower efficiencies than stamens and pistils have been obtained, the use of unopened leaves (i.e. between 1.5 to 5.0 mm long) placed abaxial side down on Petri dishes supplemented with solid NB2 medium leads to the generation of PE masses that later will regenerate into whole plants in ´Superior Seedless´, ´Thompson Seedless´ and ´Freedom´ genotypes (Dhekney *et al.*, 2009). Alternatively, convenient procedures to introduce material from the field have been reached by proper cultivation of grapevine sterile buds in solid C2D4B medium (Araya *et al.*, 2008; Gray, 1995) by one to four months and then culturing the processed tissues into NB2 solid medium.

The use of induction media based on modified MS or Nitsch (described by Li *et al.*, 2008), established the basis for additional improvements in grapevine SE. This time, authors were focused on the yield of the system. Solid cultures are heterogeneous and diffusion-limited; on the other hand, agitated liquid cultures, involve mainly faster, more uniform, efficient, and controllable mass transfer processes. It is accepted that use of liquid cultures offers numerous technical advantages over solid cultures (Archambault *et al.*, 1994). However, the actual evaluation of embryogenic protocols must be carried out on the basis of volumetric productions, true plant organ (i.e. torpedo shape, Jayasankar *et al.*, 2003), system homogeneity, and finally, conversion of embryo cells into whole plants (Archambault *et al.*, 1994). In ´Thompson Seedless´, application of an induction period by six weeks using Li´s

modifications generated PE masses that are transferred into maintenance liquid media based on B5 major salts (Gamborg *et al.*, 1968) and vitamins from MS. These flask assays have been the basis for the generation of an air-lift bioreactor as recently described Tapia *et al.* (2009). The system was designed to improve biomass production of ´Thompson Seedless´ somatic embryos and, at the same time, enabled a preliminary characterization of cell´s behavior during the grapevine SE time-course. The very first improvement derived from the use of liquid systems was that biomass multiplication rate decreased from 60 to about 40 days due to the use of agitated flasks (Figure 2a). Even better, the use of an air-lift bioreactor improved this rate to seven days (Tapia *et al.*, 2009). In addition, a lower than expected sugar consumption was observed during the SE process, suggesting side roles for this substrate during culturing. Li´s procedures described that flasks culturing leaded to the generation of up to 400 mg of biomass, obtaining PE and E cells; in those experiments, bigger inocula leaded to explants' oxidation. On the contrary, the 2 L vessel's reactor (Figure 2b) regularly admitted 2 g inocula without affecting the process, including explants viability and duplication of this biomass at the seventh day of batching. Genetic transformation procedures of somatic embryos obtained from this system did not show any difference compared to explants produced by the regular solid media-based system (depicted in Figure 1), generating fully regenerated transgenic plants (Tapia *et al.*, 2009).
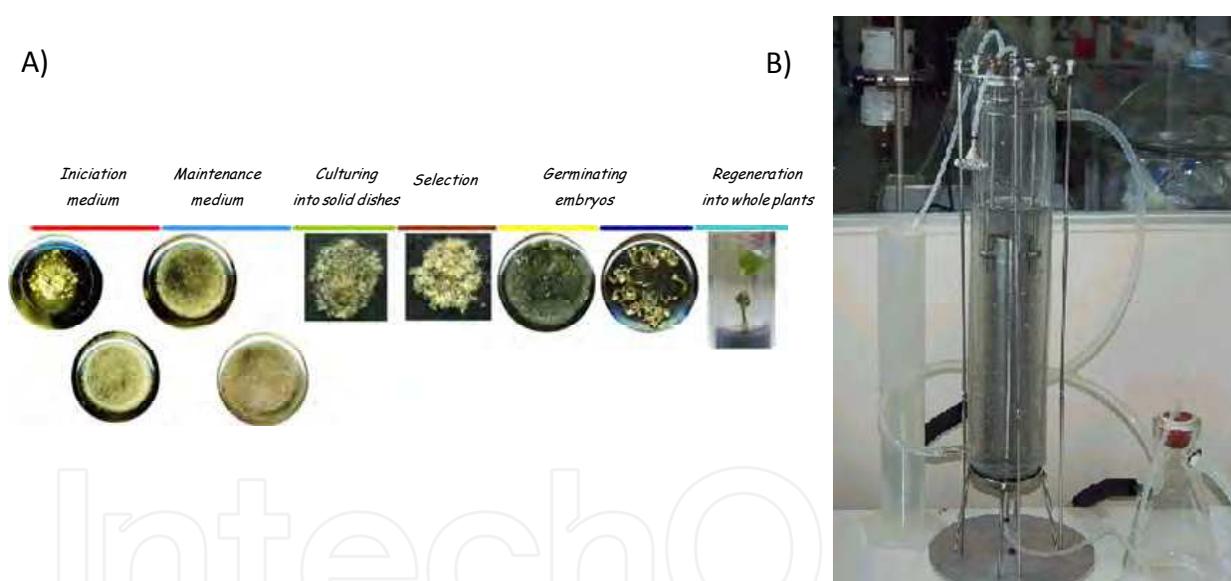

Fig. 2. Improved pipeline for somatic embryogenesis and *Agrobacterium*-mediated genetic transformation of grapevine ´Thompson Seedless´ and ´Princess´ using liquid media (A) and an air-lift bioreactor (B). Main steps are indicated.

2.4 Inducing somatic embryogenesis in recalcitrant germplasms. New strategies

Embryogenic competence can be considered as an exception more than a rule. Several genotypes have shown low or null responses to protocols that have been successfully evaluated in certain varieties. An optimized SE procedure for ´Thompson Seedless´ (Figure 3a) is not as efficient as applied on ´Red Globe´ (Figure 3b). Analyses of factors affecting competence have been recently reported (Dhekney *et al.*, 2009) by exploring changes in the induction phase. The use of MS and Nitsch macro- and micro-elements supplemented with

6-bencyl aminopurine (BAP) at 4.5 and 8.9 µM plus 2,4-dichlorophenoxy acetic acid (2,4-D) at 8.9 and 4.5 µM, respectively, leaded to most of the 18 evaluated cultivars and eight *Vitis* hybrids, to produce SE to some degree in that work. In that line, from the learning process to design and build an air-lift system, the use of liquid media offered important data about the effect of different nutrients applied in closer contact with grapevine PE cells. These findings have leaded to the use of mixed (i.e. having solid and liquid steps) protocols on diverse low-competence backgrounds such as ´Freedom´ or ´Red Globe´.

Fig. 3. Differential results of SE procedures in ´Thompson Seedless´ and ´Red Globe´.
A, An efficient SE system in ´Thompson Seedless´ generating PE and E cells (right picture, whitest cells); B, the same system applied on ´Red Globe´ showing an inefficient result.

The new strategies include the above referred use of *in vitro* leaf explants as a source of starting material for SE. Multiple *in vitro* buds are obtained from *in vitro* leaves using C2D4B (Araya *et al.*, 2008; Gray, 1995) by at least 30 days of cultivation. When these buds are transferred to solid NB2 media supplemented with 2,4-D (4.5 µM) and BAP (1 µM) by additional 120 days, PE masses are generated. These masses are the pivot for a new branch of procedures in which solid and liquid cultures are used. In ´Thompson Seedless´ or ´Princess´, bud production by culturing of leaves in C2D4B media prepare cells that will produce as many somatic embryos as required during the phase of cultivation in NB2 medium; these cultivars describe high yield enough to accomplish considerable number of transformation experiments (Dhekney *et al.*, 2009) (Figure 3a). However, such yield is not observed when the strategy is applied on ´Harmony´, ´Freedom´ or ´Red Globe´ genotypes (Figure 4a). Although very few somatic embryos are prone for transformation purposes from these materials (see whitest cells in the right picture on Figure 3b), the strategy leads to the production of a considerable remaining material, which in our hands was formerly discarded for transformation assays (Figure 4a). After several trials for recovering these masses and re-convert them into SE competent cells, a highly productive cycle was observed by culturing them solid X6 media and the addition of a liquid pulse of MS modified medium supplemented with glutamine (400 mg/L) and kinetin (4.6 µM). Under such treatments, cells are propagated, disintegrated into minimal groups of cells and leaded to

new developmental stages; as a result, the generation of an extremely high amount of somatic embryos after 30 days of treatment is achieved (Figure 4b). Although these procedures have fixed the competence for SE in these genotypes, it remains to be evaluated if such combined treatments could help this process in other grape genotypes. In the meantime, genetic transformation of grapevine rootstocks is now plausible and efforts evaluating genes related to root-linked disorders can be now evaluated.
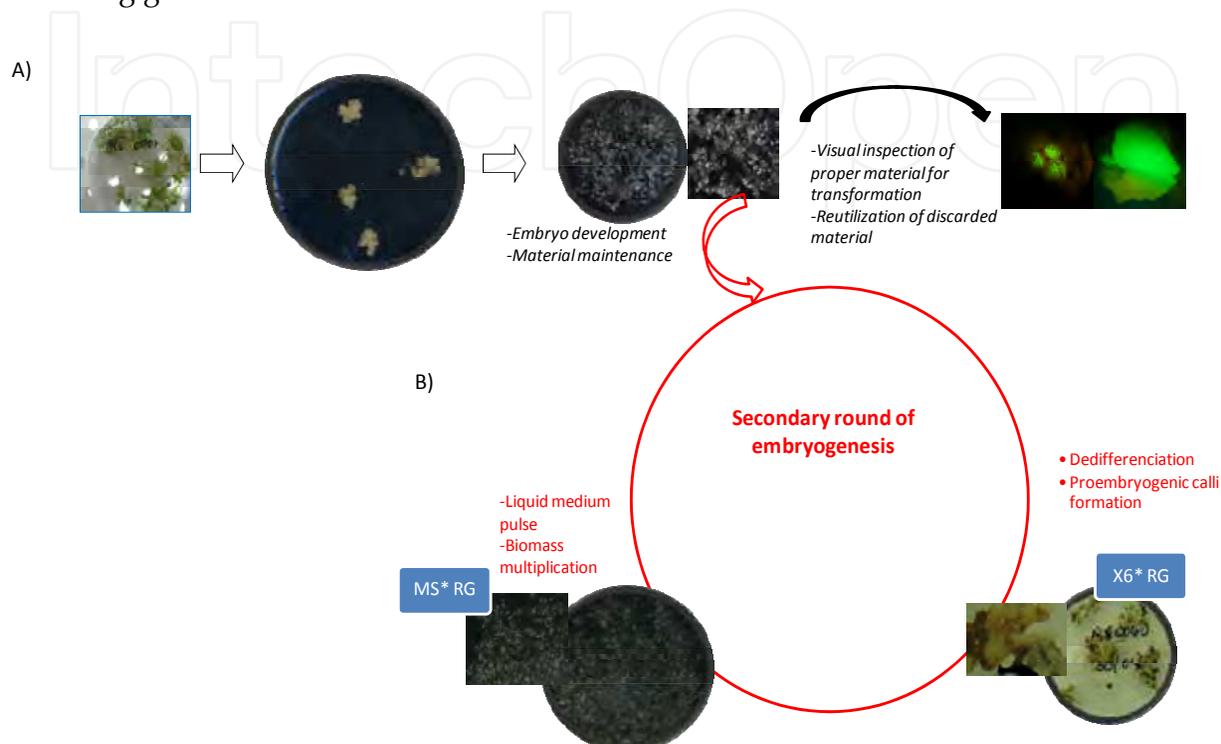

Fig. 4. Improved procedures to induce somatic embryogenesis in ´Red Globe´. The low efficiency protocol to induce and transform embryos (black arrows, A) leads to the formation of remaining cells that can be incorporated into a new round of SE based on the use of glutamine and kinetin (red circle in the diagram, B) for yield improvement.

3. Genetic transformation of plums in the waiting for a peach transformation system

It has already been almost 20 years since the generation of the most highlighted event in the field of stone fruits genetic transformation: the *Plum Pox Virus* (PPV) *Prunus domestica* resistant line named C5. Obtained by transformation of ´Bluebyrd´ explants, C5 was derived from transformation events using *A. tumefaciens* strain C58/Z707 containing a binary plasmid with the coat protein (CP) PPV gene plus the 3' non-translated region of the viral genome (EMBL Accession No X16415, Teycheney *et al*., 1989). The high degree of resistance observed has been stable in more than 10 years of evaluations in greenhouses and in the field (Hily *et al*., 2004; Malinowski *et al*., 2006; Polák *et al*.; 2008). In C5, the clone has exhibited resistance associated to RNA interference (RNAi), i.e. a high level of transgene transcription in the nucleus associated to a low level of mRNA in the cytoplasm, whereas the genetic analysis has revealed multicopy transgene insertions with repeated sequences in the presence of additional one or more aberrant copies (Scorza *et al*. 2001). RNAi in C5 was

later confirmed by detection of small interfering RNAs (siRNAs) homologous to PPV sequences (Hily *et al.*, 2005); siRNAs of 22 and 25-26 nucleotides in length were found in challenged C5 plants, whereas short siRNAs were found exclusively in wild type infected *P. domestica* controls. Results suggested that the high level virus resistance in C5 is connected with the production of this long-sized class of siRNAs (Hily *et al.*, 2005). Recently, multicopy arrangements of T-DNA fragments from the transformation plasmid in the C5´s genome have been determined, showing the occurrence of an *in planta* hairpin structure of the introduced CP gene (Kundu *et al.*, 2008; Scorza *et al.*, 2010) and explaining the possible source for RNAi in C5.

Multiple tissues have been used for plant regeneration in the *Prunus* genus, including leaves (Gentile *et al.*, 2002; Yancheva, 2002; Dolgov *et al.*, 2005), cotyledons (Mante *et al.*, 1989), embryos (Pérez-Clemene *et al.*, 2004), and hypocotyls (Mante *et al.*, 1991; Gonzalez-Padilla *et al.*, 2003). As a system, generation of C5 represents to date the more successful and reproducible procedure for plant regeneration in this genus, obtained by the use of medial hypocotyl segments (Mante *et al.*, 1991). To get this procedure, 1-phenyl-3-(1,2,3-thidiazol-5-il) urea (thidiazuron, TDZ) at 7.5 µM and indole-3-butyric acid (IBA) at 0.25 µM were applied to the explants in order to regenerate *Agrobacterium*-mediated transformed hypocotyl segments. The hypocotyls based system was later improved by González-Padilla *et al.* (2003); these authors described a reduction in the total number of regenerated shoots without affecting the transformation rate by application of early selection (80 mg/L of kanamycin and 300 mg/L of Timentin) during shoot regeneration in a medium consisting on half-strength MS salts and vitamins supplemented with 5 mM α-naphthaleneacetic acid (NAA) and 0.01 mM kinetin.

From these works, new improvement was made in the diploid species *Prunus salicina*. The regeneration and genetic transformation of Japanese plum using the approach of hypocotyl segments described in *P. domestica* was described by Urtubia *et al.* in 2008 (Figure 5). Previously, Tian *et al.* (2007) used a constant concentration of TDZ (7.5 µM) that eventually was combined with variable amounts of BAP (2.5 or 12.5 µM) or kinetin (12.5 µM) to induce shoot formation in ´Shiro´ and ´Early Golden´; additional evaluation of ´Redheart´ hypocotyl segments did not generate plantlets by this protocol. In successful genotypes, results demonstrated that the use either of TDZ or of TDZ plus BAP have allowed the establishment of whole plants acclimatized at greenhouse level. For transformation setting up of this species, Urtubia *et al.* (2008) described the use of different TDZ/IBA ratios (6:1 to 10:1) to regenerate *Agrobacterium* transformed ´Angeleno´ and ´Larry Ann´ hypocotyl segments. The shooting on average on 12% of the total cultured explants and the establishment of whole plants expressing the green fluorescent protein in the greenhouse concluded this research and established Japanese plum as a model diploid species in *Prunus* transformation. Despite this, the observed varietal dependence of results and the low number of confirmed positive transgenic lines ratified the difficulty to obtain genetic transformation systems in stone fruit species. Authors also described the effect of using different *Agrobacterium* strains on the co-cultivated *P. salicina* explants; whereas the use of LBA4404 strain leaded to significant oxidation in the treated explants, infections with EHA or GV strains led to the production of whole plants with no major disadvantages throughout the protocol.
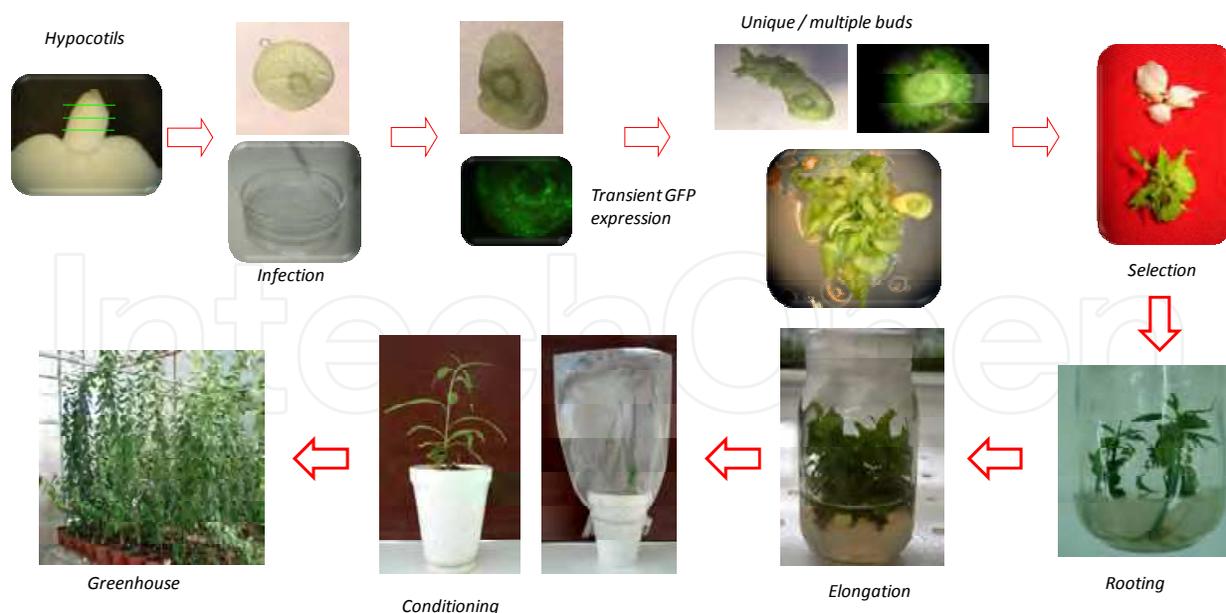
Fig. 5. ´Angeleno´ Japanese plum genetic transformation procedures. The complete pipeline for *Agrobacterium*-mediated genetic transformation of *P. salicina* is showed.

3.1 A new example: RNAi-based PPV resistance in Japanese plums

Holding the research focus on PPV, new strategies against the virus can be evaluated with improved expectations, including new technology development obtained for gene silencing. As mentioned, an additive effect of multicopy T-DNA fragments arranged in C5´s genome and the occurrence of an *in planta* hairpin structure for the introduced CP gene (Kundu *et al.*, 2008; Scorza *et al.*, 2010) onset an adequate silencing scenario in the plant. Hairpin RNA inducing DNAs have been described as an important activator of RNAi in transgenic plants (Watson *et al.*, 2005). Artificial *in vivo* generation of siRNA was soon published by introduction of hairpin RNA inducing plasmids into target cells (Wesley *et al.*, 2001); since then, multiple findings have been focused on the development of optimal constructs to generate siRNAs in transgenic plants (Watson *et al.*, 2005; Qu *et al.*, 2007), and making gene silencing through RNAi one of the most promissory strategies to boost plant immunity nowadays (Pennisi, 2010).

Recently, Dolgov *et al.* (2010) developed and used a coat protein gene-based hairpin inducing plasmid to generate RNAi transgenic *P. domestica* L. 'Startovaya' plants. This group had previously generated a transformation protocol for European plum using adult tissue-derived explants by use of 5-12 days old leaves (Mikhaylov and Dolgov, 2007). This alternate transformation strategy involves slight wounding of explants and the application of a five hours auxin shock in liquid MS medium supplemented with indole-3-acetic acid (28 µM). After co-cultivation procedures, regeneration is induced by culturing in MS modified salts including BAP (22 µM) and IBA (1.96 µM) and the presence of convenient selection antibiotics (hygromycin) plus cefotaxime to keep *A. tumefaciens* AGL0 used in the process under control. Elongation of treated explants is obtained by use of the same growth regulators in lower quantities than used for regeneration (BAP 8.8 µM, IBA 0.5 µM) and the inclusion of a 16 h photoperiod. As preliminary results of transformed individuals challenged by grafting experiments, these authors have indicated the potential development of five candidate lines that challenged by grafting on PPV-infected rootstocks demonstrated successfully disease resistance with no virus accumulation as suggested the use of Western blotting experiments.

Considering that genome annotation for peach is expected in the very near future, the advantage of transforming a diploid species in the *Prunus* genus opened space for candidate gene evaluation in this type of fruit trees, making very attractive the use of Japanese plums as a closer model. The approach of silencing PPV sequences has been under development in *P. salicina*; an arrangement of "hot-spot" target sequences from the coat protein gene and predicted as highly sensitive to gene silencing, have been located in tandem in a construct designed to generate *in vivo* RNA hairpin. The use of this construct for genetic transformation of Japanese plum hypocotyls leaded to the generation of six transgenic lines that putatively could silence the PPV coat protein gene. Acclimatized and rooted transgenic plants harboring this construct were used has a source of scions for grafting experiments on PPV-infected *Prunus insititia* rootstocks that previously had been used for a confined evaluation of C5 scions (Wong *et al.*, 2010). Symptomatology (Figure 6) and ELISA data from the first season of evaluations for these lines have indicated the existence of two transgenic lines showing the recovery and resistant phenotypes, respectively (Figure 6). Massive sequencing data is under development for these lines in order to corroborate small RNAs populations generated in these challenged ´Angeleno´ scions.
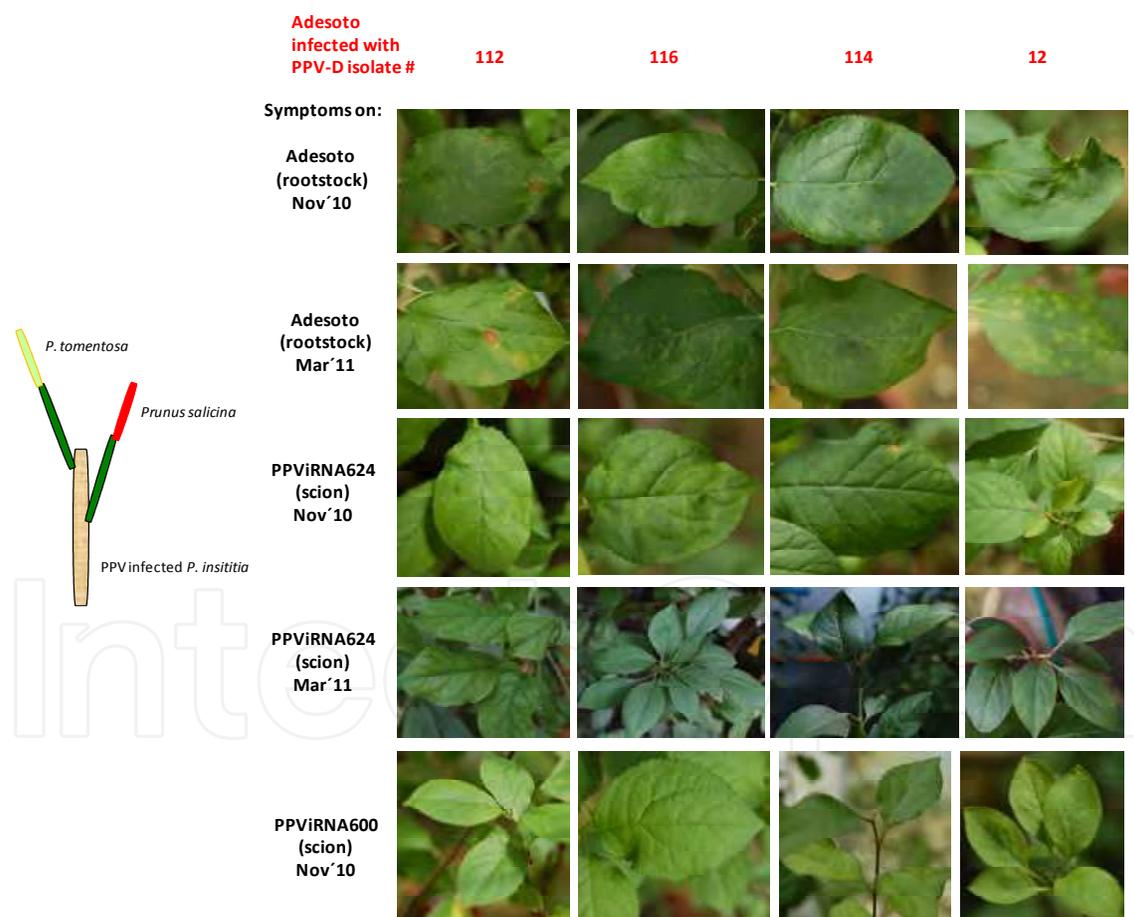

Fig. 6. Genetically modified ´Angeleno´ plants challenged by micro-grafting onto previously infected 'Adesoto' rootstocks. Four different PPV-D Chilean isolates (numbers) have been evaluated.

These assays are carried out in a biosafety greenhouse at La Platina Station of the National Institute of Agriculture, Santiago, Chile.

4. Towards peach genetic transformation

In parallel to the systems developed for plum, the race for a *Prunus persica* genetic transformation procedure has been run with not such a clear achievement. Peach highlights as one of the more recalcitrant species regarding the *in vitro* organogenesis process. First attempts for tissue culture in peach were focused on propagation (Hammerschlag, 1982). From these studies, some factors affecting the establishment of apical buds were identified, such as disinfection procedures for introduced material from the field, plant growth regulators applied and temperature of culturing. Also, as observed in grapevines, genotype-dependent responses were obtained mainly in the rooting and acclimatization steps during *in vitro* establishment. Authors suggested that endogenous hormone variations were responsible for these differential responses obtained at culturing (Hammerschlag *et al.*, 1987). In 1989, da Câmara Machado *et al.* gave patterns for bud generation using leaf discs and micro-sticks from some apple, pear and peach cultivars to address *Agrobacterium*-mediated genetic transformation of these species. Results from such studies showed just slight callusing leading to rare budding in the cutting edges of the explants with no regeneration. In the same period, other works reported poor bud generation from *P. persica* explants such as immature endosperms (Meng and Zou, 1981), zygotic embryo-derived calli (Hammerschlag *et al.*, 1985; Bhansali *et al.*, 1990; Scorza *et al.*, 1990a), immature (Mante *et al.*, 1989) and mature (Pooler and Scorza, 1995) cotyledons.

Most of the studies describing genetic transformation in peach have described the use of *A. tumefaciens* infection in either immature or mature explants (Hammerschlag *et al.*, 1989; Scorza and Sherman, 1996; Pérez-Clemente *et al.*, 2004; Padilla *et al.*, 2006); in general, targeting on immature tissues leads over the use of mature explants in woody species (da Câmara Machado *et al.*, 1992; Ye *et al.*, 1994). In those experiments, the use of cytokinines (BAP, kinetin, zeatin or isopentyladenine) has been reported in order to induce regeneration of immature cotyledons, although the number of produced buds has been extremely low. Pérez-Clemente *et al.* (2004) reported the use of mature cotyledons with a very low adventitius budding efficiency. No whole plants or stable foreign DNA incorporation into the tissues were described from these reports. In addition, Pérez-Clemente *et al.* (2004) proposed the use of longitudinal embryo segments as a reliable method for regeneration and plant genetic transformation, however, massiveness and reproducibility of this methodology has not been really achieved.

Transversal stem segments isolated from mature plants were infected with the *shooty Agrobacterium* mutant (tms328::Tn5; Hammerschlag *et al.*, 1989), which commonly induces budding in tobacco calli. A cytokinine-independent growth was observed in the treated tissues, although no budding was finally observed. In a similar strategy, in which stems were replaced by immature embryos, Smigocki and Hammerschlag (1991) reached regeneration of a few buds from calli generated from embryos; however, a marked trend to rooting of the treated explants and no transgenic status of the obtained buds was reported by the authors. Recently, Padilla *et al.* (2006) described optimal conditions for gene transfer using *Agrobacterium* in different explants types (hypocotyls cylinders, cotyledons, embryonic axis from no germinated seeds, internodal segments isolated from epicotyls obtained from non germinated seeds). Initially, authors established transient expression rates for different DNA constructs and *Agrobacterium* strains, determining that cotyledons and nodal segments were the explants showing the highest β-glucuronidase and green fluorescent protein (GFP)

transient expression. These observed responses were variety dependent and none of these explants leaded to fully regenerated plants.

4.1 Combining trials to obtain genetically modified peaches

As depicted, explants from *P. persica* excepting a couple of examples referred to embryo longitudinal segments and mature cotyledons, the species can be considered as recalcitrant to *in vitro* regeneration. In addition, these successful responses do not represent a reliable process applicable to several other varieties or hybrids.

In our hands, the use of leaves as starting explants in the transformation pipeline for ´O´Henry´ did not produce a consistent number of buds, on the contrary to the reported by Gentile *et al.* (2002). Despite this and after several years of evaluations, the use of immature cotyledons leaded us to propose a combined procedure based on these procedures for regeneration and transformation of this and other commercial cultivars. ´O´Henry´ and ´Rich Lady´ immature cotyledons have been cultivated in LP modified medium (Gentile *et al.*, 2002) generating viable explants that can produce either direct budding in MP modified medium (Gentile *et al.*, 2002) supplemented with BAP (5 µM) and NAA (concentrations between 3 and 5 µM) or lead to formation of white structures (as shown in Figure 7) that, in presence of LP medium and 2,4-D (1 µM), will become into green buds after 60-90 days of culturing.

Fig. 7. *Prunus persica* regeneration system. A brief and summarized scheme of the system for peach regeneration using immature cotyledons.

These results are quite similar to those obtained using mature cotyledons from *Prunus avium* and the technical approach is the same (Canli and Tian, 2008). Activated tissues can be then cultured in LP medium supplemented with 2,4-D (1 µM) and BAP (3 µM) for additional 90 days and then transferred into LP salts with no growth regulators to obtain gradually green buds which can be transferred into a LP derivative medium supplemented with BAP (0.5 µM), IBA (0.01 µM) and glutamine (0.2 g/L). Shoots start to appear after eight months of total treatments and generation of plantlets can be reached after 8-12 months. Up to date, in the most responsive cultivars (i.e. 'O'Henry' and 'Rich Lady'), regeneration rates are close to 20% and transformation efficiencies in the regenerated plantlets are close to 2%.

By this methodology, trees expressing GFP have been generated (Figures 8 and 9); interestingly, the behavior of a 35S RNA Cauliflower Mosaic Virus promoter driving the *gfp* gene expression could be analyzed (observed) in peaches by epifluorescence microscopy in leaves, fruit tissues (mesocarps), and radicles (Figure 8). The same qualitative analysis carried out in flowers from these trees (Figure 9), have shown clear differences in the fluorescence obtained when transformed tissues are compared to the corresponding non transgenic controls.
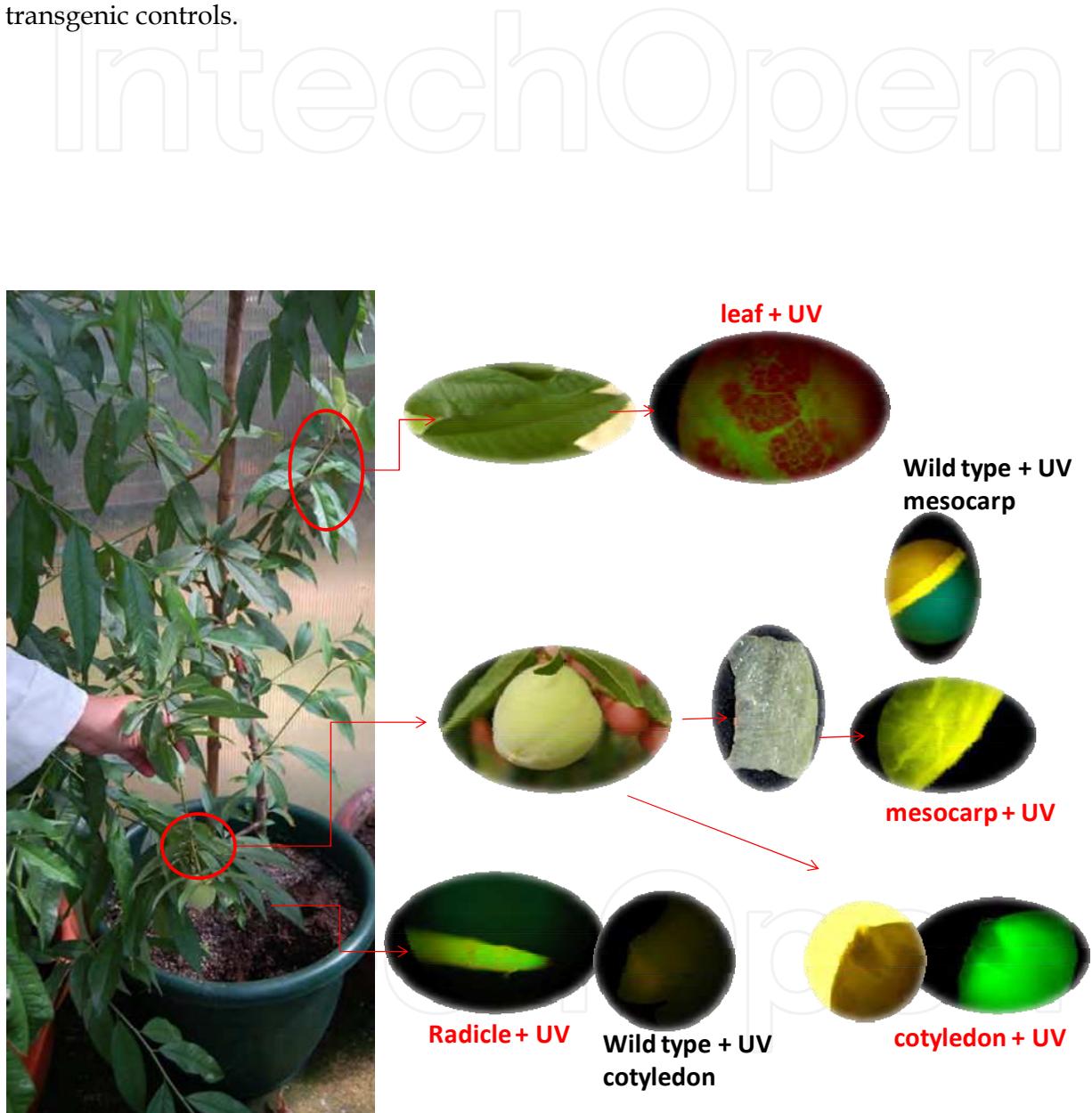
Fig. 8. Genetically modified peach tree expressing GFP.
Epifluorescence microscopy in different tissues from a genetically modified peach tree. Red fonts indicate view of transgenic tissues under UV light (+UV); black fonts show views of the corresponding tissues from non transformed trees used as a control.
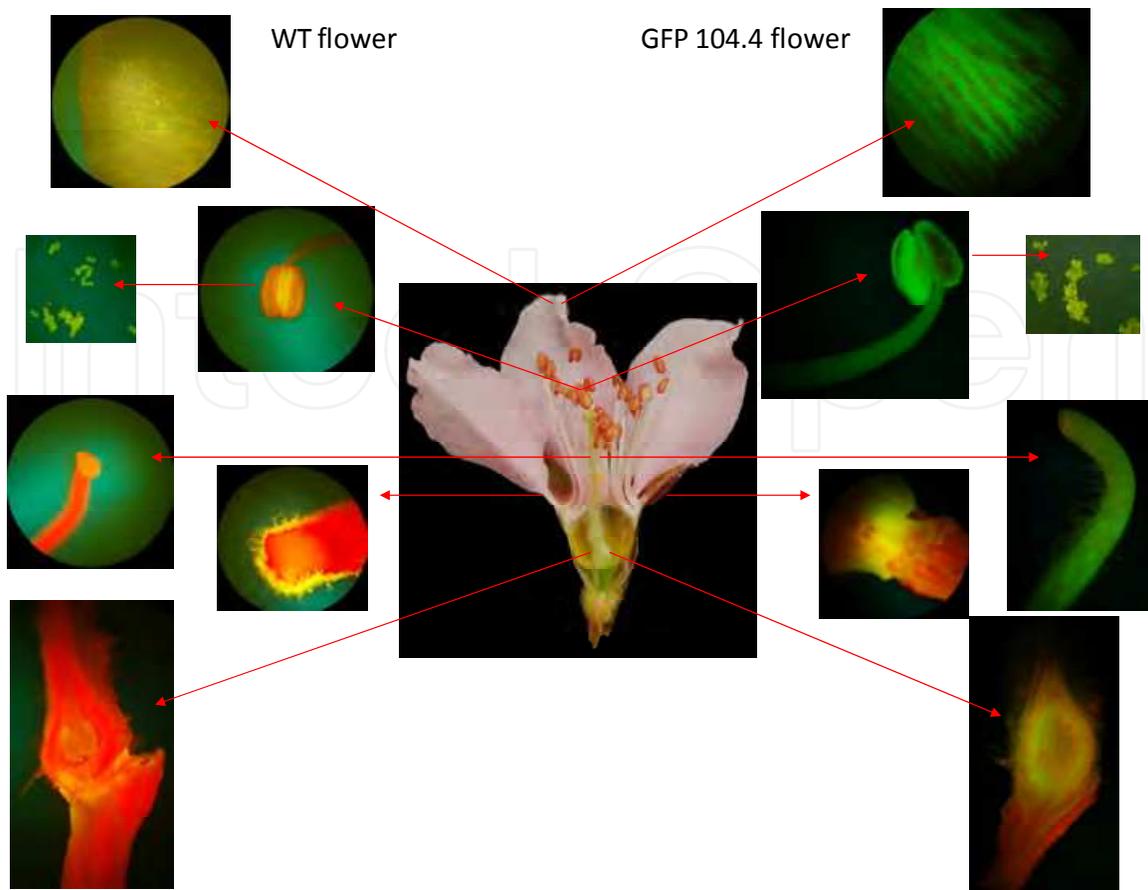
Fig. 9. Schematic representation of different floral organs from a genetically modified peach flower expressing GFP (right, GFP 104.4 flower) compared to wild type tissues (left, WT flower).

5. Conclusions

A compact view about different strategies to reach successful genetic transformation experiments in grapes and stone fruits (plums and peach) has been presented using results from our close experimentation. This time, the focus has been on mixing different ideas from procedures used in both genera and their corresponding results. *Vitis* spp. genetic transformation has mainly relied on the use and availability of SE procedures. Early in the ´70s, regeneration of whole Gloryvine (*V. vinifera* x *V. rupestris*) plants was achieved from somatic embryos previously obtained in this hybrid by anthers tissue culture cultivation. Since then, successful procedures have made possible SE establishment in *V. vinifera*, *V. labruscana* and *V. rotundifolia*. Recently, mixing of solid and liquid media-based procedures, grapevine SE has leaded to the design, characterization and scaled-up production of ´Thompson Seedless´ embryogenesis using an air-lift bioreactor. The system can be expanded to other genotypes. The accumulated information derived from this scaling-up process fused to the characterization of some of the kinetic parameters involved in grapevine SE, have enabled design of new experimentation focused on the development of SE protocols for genotypes such as rootstocks (´Harmony´, ´Freedom´ or ´Salt Creek´) and more recalcitrant cultivars such as ´Red Globe´. The results indicate that grape genetic transformation can be considered as a model system in which efficiency is not necessarily an issue and the possibility for high through-put candidate gene evaluation is plausible.

In case of *Prunus* spp., European and Japanese plums have been the more successful rosaceous fruit models to be regenerated and transformed in our hands (plums and peach). Under the trade name HoneySweet and deregulated in 2009, the PPV-resistant C5 event established a baseline for regeneration protocols using seed-derived tissue explants; these were successfully dissected and evaluated on *P. salicina* using four relatively close media. The regenerative responses, with shooting on about 12% on the total cultured explants in two varieties (´Larry Ann´ and ´Angeleno), leaded to the generation of stable genetically modified lines. The research "baseline" in plums transformation has later conducted to improving regeneration and transformation efficiencies in both species, from which new PPV-resistant plant materials have been produced. Hairpin dsRNA inducing constructs are currently under evaluation for silencing different PPV genes, from which CP has been here illustrated. Finally, these achievements have been recently optimized, reaching the successful transformation of European plum leaf explants in some genotypes. Plum can be considered as an attainable model system for candidate gene evaluation in stone fruits, with hexaploid and diploid versions for such studies. At the same time, plum genetic transformation can be judged as a proof of concept for peaches.

For *P. persica*, genetic transformation seems attainable although not reproducible. Several protocols have worked just in the place when they were generated. In our hands, the use of immature cotyledons subjected to modifications in the workflow described for leaves regeneration has allowed for generation of GFP expressing peaches. Consistently, this platform has leaded to the production of new transgenic lines and constructs already evaluated in Japanese plum (i.e. PPV silencing), are now used for this species. The peach case reinforces the concept that one previous development is a necessary step leading to the next one.

6. Acknowledgments

7. References

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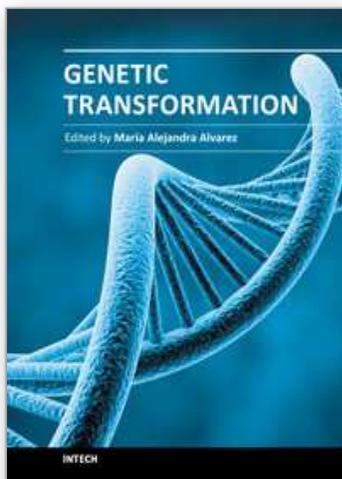
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Edited by Prof. MarÃa Alvarez

Genetic transformation of plants has revolutionized both basic and applied plant research. Plant molecular biology and physiology benefit from this power fool, as well as biotechnology. This book is a review of some of the most significant achievements that plant transformation has brought to the fields of Agrobacterium biology, crop improvement and, flower, fruit and tree amelioration. Also, it examines their impact on molecular farming, phytoremediation and RNAi tools.

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