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Breeding in peach, cherry and plum: from a tissue culture, genetic, transcriptomic and genomic perspective

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ABSTRACT

This review is an overview of traditional and modern breeding methodologies being used to develop new *Prunus* cultivars (stone fruits) with major emphasis on peach, sweet cherry and Japanese plum. To this end, common breeding tools used to produce seedlings, including *in vitro* culture tools, are discussed. Additionally, the mechanisms of inheritance of many important agronomical traits are described. Recent advances in stone fruit transcriptomics and genomic resources are providing an understanding of the molecular basis of phenotypic variability as well as the identification of allelic variants and molecular markers. These have potential applications for understanding the genetic diversity of the *Prunus* species, molecular marker-assisted selection and transgenesis. Simple Sequence Repeat (SSR) and Single Nucleotide Polymorphism (SNPs) molecular markers are described as useful tools to describe genetic diversity in peach, sweet cherry and Japanese plum. Additionally, the recently sequenced peach genome and the public release of the sweet cherry genome are discussed in terms of their applicability to breeding programs.

INTRODUCTION

The genus *Prunus* (Rosaceae) encompasses several economically important stone fruit species such as peach, sweet cherry and plum, among others. During the last 100 years great advances have been achieved using the traditional tools of genetic improvement such as crossing, selection, statistical design, evaluation of superior lines and *in vitro* propagation of new cultivars (Hancock et al., 2008; Okie and Hancock, 2008; Iezzoni, 2008).

Most of the cherries sold for consumption are called sweet cherries and are generally from the species *Prunus avium*, while those for cooking/processing are sour cherry (*Prunus cerasus*). In the case of peaches all are of the species *Prunus persica* (nectarines and peaches). For plums there are two species, *Prunus salicina*, commonly called Japanese plum, used to prepare liquor in China and Japan and *Prunus domestica*, known as European plum, consumed as fresh or dried fruit.

Traditional genetic improvement has led to the development and commercialization of highly productive cultivars adapted to diverse biotic and/or abiotic conditions, together with good fruit quality to meet consumer demands. Since the mid-1990s, *Prunus* species (especially peach, sweet cherry and plum) have been characterized molecularly (Belthoff et al., 1993; Dirlewanger et al., 2012). Genome sequencing and new gene discovery methods such as next generation sequencing are revealing important structural and regulatory genes, as well as molecular polymorphisms associated with important agronomic traits (Verde et al., 2012; Dirlewanger et al., 2012).

Molecular breeding in *Prunus* species is combining traditional plant physiology with genetic and genomic analyses to assist traditional breeding programs as well as using alternative technologies (i.e. transgenesis) to improve these cultivars. The objective of this review is to describe the state of the art of traditional and molecular methodologies used in *Prunus* genetic improvement programs, with special emphasis on peach, cherry and plum.

I. BREEDING AND GENETICS

A. Traditional breeding

Peach, sweet cherry and plum are commercially important *Prunus* species (Okie and Weinberg, 1996; Scorza and Sherman, 1996; Sansavini et al., 2006). Although these fruit species have been cultivated for some 2,000-4,000 years, but it was only only during the last 100 years that breeding programs began to develop new cultivars (Okie and Hancock, 2008; Iezzoni, 2008). According to Byrne (2005), approximately 3,000 cultivars of *Prunus* species have now been released from breeding programs. Currently, modern breeding programs for peach, sweet cherry and Japanese plum are focused on satisfying both consumer and grower preferences. Consumer preferences are related mainly to fruit quality and so breeding programs are directed towards obtaining fruits that have acceptable taste and desirable texture. On the other hand, growers require highly productive cultivars, resistance to disease, different harvest dates to prolong the period of fruit production and high storability (Byrne, 2005).

The breeding techniques used to develop new peach, sweet cherry and plum cultivars (Table 1) have similar methodologies. Crossing strategies applied during breeding programs are mainly hybridization (intra and interspecific) and open pollination. Peach is easily self-pollinated since it is self-compatible. However, many Japanese plum and sweet

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cherry genotypes are self-incompatible or may be classified into incompatible groups, although some self-compatible sweet cherry cultivars have been described (e.g. "Stella", Lapins, 1970). Due to the self-incompatibility of plum and sweet cherry, the flowers do not need to be emasculated when hybrids are being produced. However, emasculation is indispensable for peach in order to avoid self-pollination. More than 60% of the cultivars released have been developed using crossing and selection methodology; the remaining cultivars has been produced by clonal selection following open pollination or induced mutations (Scorza and Sherman, 1996).

Other breeding aspects, such as pollen management and seedling production (conventional seed germination and embryo rescue), also use similar protocols (Table 1). The number of seedlings produced per year depends on the needs of each breeding program. Japanese plum and peach breeding programs usually produce 3,000 to 4,000 seedlings per season. Sweet cherry programs do not produce such high numbers of seedlings per season, mainly due to difficulties with seed germination (Brown et al., 1996; Iezzoni, 2008).

Comparing breeding programs of these stone fruit species, the length of the juvenile period (from seed to flower) is very different. Peach seedlings only take 1 to 2 years to produce their first flowers, while Japanese plums and sweet cherries produce their first flowers only after 2-3 years and 3-5 years, respectively (Scorza and Sherman, 1996; Okie and Hancock, 2008; Iezzoni, 2008). The length of the juvenile phase clearly affects the breeding cycle, particularly for fruit evaluation, selection of the best hybrids and cultivar release (Table 1).

B. Genetics

As in all *Prunus* species, peach, Japanese plum and sweet cherry have a basic chromosome number x=8 and the diploid genome organized in 2n=2x=16 chromosomes. Peach is a highly genetically characterized fruit tree and at the moment is considered a model species for the family Rosaceae (Monet and Bassi, 2008; Arús et al., 2012).

Genetic studies of some pomological traits have been carried out during the last 15 years; consensus genetic maps have been built based on intraspecific and interspecific crossing of peach (Abbott et al., 2007); also several transcriptomic and genomic studies have reported genes controlling a large number of important fruit traits (Dirlewanger et al., 2012). Verde et al. (2013) recently published a high-quality draft genome of peach which is a baseline for comparative analysis with other *Prunus* species as well as other trees.

The inheritance pattern of some traits associated with fruit quality and productivity (taste, texture, size, shape, flesh and external color, firmness, shelf life, yield, bloom date and harvest date, among others) have shown a quantitative pattern of inheritance. High heritability values have been reported for peach, plum and sweet cherry for flowering date (h^2 = 0.67-0.94); ripening date (h^2 = 0.44 -0.99) and fruit size (h^2 = 0.5 – 0.68), and medium to low values for browning (h^2 = 0.35); acidity (h^2 =0.19-0.31); soluble solids (h^2 = 0.17 – 0.36); sweetness (h^2 = 0.19) and flavor (h^2 =0.06-0.16) (Scorza and Sherman, 1996; Okie and Weinberg, 1996; De Souza et al., 1998; Fotric et al., 2007; Hancock et al., 2008; Okie and Hancock, 2008; Dirlewanger et al., 2012). Nevertheless, there are only some few studies about the inheritance pattern and the genes that control physiological disorders (e.g. woolliness and internal breakdown) expressed in peach and nectarine flesh after cold storage. Ogundiwin et al. (2007, 2008) suggested that polygenic control was involved in their expression. Endopolygalacturonase and leucoantocyanidin dioxygenase have been suggested to be involved in these post-harvest disorders. Additionally, Peace et al. (2006) pointed out that internal breakdown in peach has high heritability and also suggested that endopolygalacturonase played an important role. Also, Gonzalez-Aguero et al. (2008) found that the expression level of the cobra, endopolygalacturonase, cinnamoyl-CoAreductase and rab11 genes were lower in juicy fruit compared to woolly fruit.

The inheritance patterns of other traits has shown Mendelian behavior. Dominance has been reported for red flesh over yellow flesh and yellow flesh over white flesh. Low malic acid is dominant over normal level, peach over nectarine, freestone over clingstone, melting flesh over non-melting flesh and normal ripening over slow ripening ("stony hard", scarce ethylene production) (Scorza and Sherma, 1996; Haji et al., 2005).

C. Genetic diversity

Obtaining information about genetic diversity and structure in *Prunus* species can provide useful genetic information for crop breeding and genetic research. Today, molecular markers

TABLE 1

Main aspects of plum, cherry and peach genetic improvement

	Plum	Cherry	Peach
Crossing strategy	H, OP	H, OP	H, OP, S
Emasculation	no	no	yes
Pollen drying (°C/hours)	22/12	22/12	25/12
Seed germination by conventional stratification (°C/days)	2-4 /90-120	0-5 /90-180	2-4/90-120
Embryo rescue plus stratification (°C/days)	2-4 /70-75	5/60-120	2-4/75
Seedling production (seedling /year)	3,000-4,000	360-420	3,000-4,000
Time to reach the first flowering (years)	2-3	3-5	1-2
Time to cultivar release (years)	15-18	18-20	10-12

H = Hybridization; OP = Open pollination; S=Self-pollination.

have enabled the organization of genetic diversity for wild and cultivated genotypes to be described. Simple sequence repeats (SSR or microsatellites) are tandem repeats of 1–6 bases. They are commonly used in population genetic studies because they are widely distributed throughout the genome, co-dominant and highly polymorphic. They have been isolated in different species of the family Rosaceae and are widely used for such aspects as genetic diversity analysis, cultivar identification, fingerprinting and mapping genetic linkage (Aranzana et al., 2003; Abbott et al., 2007; Fotiric et al., 2007).

The most recent studies carried out in peach, sweet cherry and plum have shown important differences in genetic diversity associated with the breeding system and genetic improvement. Plum and sweet cherry are allogamous species because they are self-incompatible, a favorable condition for outcrossing between unrelated individuals. Also, they show high levels of heterozygosity for SSRs. In contrast, peach (including nectarines) is self-compatible and predominantly autogamous (Hegedüs et al., 2006), thus low levels of heterozygosity are expected (Bouhadida et al., 2011).

Table 2 is a compilation of some statistics related to variability in old and modern cultivars of peach, sweet cherry and plum. Peach shows intermediate values of H_o compared to sweet cherry and plum (Table 2). In peach and nectarine, the number of alleles per locus ranges between 2.9 and 7.3, with observed heterozygosity (H_o= 0.21-0.46) consistently inferior to the expected heterozygosity (H_e=0.41-0.66), indicating a deficit of heterozygotes and a significant level of inbreeding (F= 0.08-0.53). These results are not unexpected, as noted above, since in peach 90% of fruit set is from self-pollination (Szabò and Nyéki, 1999; Monet and Bassi, 2008). However, inbreeding values observed in peach (Table 2) are significantly inferior to F values obtained for strictly self-pollinating species such as wheat (F= 0.93, Dreisigacker et al., 2005). This means that peach and nectarine cultivars still maintain an important level of heterozygosity, which can be explained by its outcrossing rate (around 10%) and breeding methodologies such as intraspecific hybridization between unrelated cultivars through crossing cycles.

In contrast, plums and sweet cherries display higher levels of genetic variability compared to peaches. The number of alleles per locus ranges from 4.1 to 12.1, the observed heterozygosity from 0.53 to 0.74, and expected heterozygosity from 0.56 to 0.8. Additionally, inbreeding rates range from -0.002 to 0.08. Self-incompatibility in plums and sweet cherries may explain in part these results. For these two species, cultivar development has been carried out only by crossing two compatible cultivars and by open pollination between a group of compatible cultivars, which would tend to produce the high level of genetic variability observed in these species.

Knowing the heritability of important agronomical traits and the pattern of genetic variability from molecular marker data will form a useful base to increase the efficiency of breeding programs in peach, sweet cherry and plum. This information will allow breeders to know how a specific trait will respond to a selection protocol and, in addition, molecular makers will make it easier to identify more genetically distant parental lines in order to maximize the genetic variability in the offspring and avoid the self- incompatibility barriers.

II. TISSUE CULTURE APPLICATIONS

Tissue culture is the aseptic culture of cells, tissues, organs or whole plants under controlled nutritional and environmental conditions (Thorpe, 2007). It is based on: i. the genetically

TABLE 2

Simple Sequence Repeat (SSR) diversity in *P. Persica (*peach), *P. salicina* (Japanese plum) and *P. avium* (sweet cherry)

Species	N	L	A	H_o	H_e	F	Place	Reference
P. persica	212	16	7.3	0.35	0.50	0.23	Spain	Aranzana et al., 2003
	94	15	6.7	0.23	0.57	0.08	Spain	Bouhadida et al., 2011
	16	18	6.9	0.46	0.66	0.33	Spain	Bouhadida et al., 2009
	117	9	6.6	0.35	0.55	0.37	Chile	Rojas et al., 2008
	27	41	4.2	0.26	0.41	0.37	France	Dirlenwanger et al.,2002
	104	53	2.9	0.21	0.45	0.53	China	Cao et al.,2012
P. salicina	8	27	5.7	0.73	0.74	0.01	Spain	Mnejja et al., 2004
	29	8	12.1	0.90	0.80	-0.13	Chile	Carrasco et al., 2012
P. avium	31	14	5.0	0.60	0.64	0.06	Lithuania	Stanys et al., 2012
	21	15	5.8	0.71	0.63	-0.03	Greek	Ganopulus et al., 2011
	278	10	7.5	0.53	0.70	0.08	Italy	De Rogatis et al., 2013
Wild	211	26	9.0	0.65	0.68	0.04	France	Mariette et al., 2010
Land race	141	26	7.6	0.66	0.64	-0.02	France	Mariette et al., 2010
Modern cvs.	66	26	4.1	0.59	0.56	-0.05	France	Mariette et al,. 2010

N = Sample size; L = Number of loci; A = Number of alleles; H_o = Observed Heterozygosity; H_e = Expected Heterozygosity; F= Inbreeding rate equal to 1- Ho/He.

retained capacity of any living cell to originate a new genetically identical cell by division and by processes of differentiation to form tissues, organs, systems and complete individuals *(cell totipotency)* (Haberlandt, 1902; Takebe et al., 1971), and ii. the capacity of plant cells to modify the response which allows them to respond to external stimuli directed towards the achievement of a determined response *(cell plasticity)* (Thorpe, 2007). There is considerable variation regarding the physiological behavior of plant tissues under *in vitro* culture conditions and this variation is often associated with genotype-dependent responses.

Tissue culture techniques have found a large number of applications in the last decades, such as: massive micropropagation of commercial plants (Sulusoglu and Cavusoglu, 2013); germplasm conservation (Isac et al., 2010); variation of ploidy levels (de Oliveira et al., 2013); development of new hybrids (Squirrell et al., 2005); development and propagation of transgenic plants (Faize et al., 2013); virus and disease elimination (Paunovic et al., 2007) and embryo rescue (Eroglu et al., 2012).

The breeding of *Prunus* species faces some technical difficulties for which *in vitro* technologies appear as a suitable solution. For example, incorporation of selected genotypes into commercial production can take several years if propagation is done by traditional methods. However, micropropagation can produce thousands of plants at a reasonable cost in a very short period of time (García-Gonzales et al., 2010). In addition, the use of meristem culture or thermotherapy can provide clean planting material (free of viruses) for new orchards, thus avoiding or reducing economic losses (Gella and Errea, 1998).

A. Achievements in micropropagation

In vitro propagation is probably the most extended application of plant tissue culture in plants (Thorpe, 2007). It may be interpreted as a simple application of basic techniques to propagate plants under aseptic and controlled conditions; however, the reality is very different because there are several difficulties associated with optimal tissue culture propagation of any species.

According to Garcia-Gonzales et al. (2010), plant propagation by tissue culture has five stages: I- *Preparation of donor plant*: where the donor plant is managed so that it has healthy and young tissues with potential to produce *in vitro* plants with no contamination; II- *Introduction and establishment:* where the tissues isolated from a donor plant are disinfected and forced to produce a morphogenic response; III- *Propagation of plants*: aimed to increase the number of individuals in the tissue culture system until the desired number is obtained by using organogenesis or somatic embryogenesis; IV- *Rooting and explant preparation for* ex vitro *conditions*: where rooting of the *in vitro* propagated plants is induced or if it does not occur, the explants are pre-adapted to the *ex vitro* environment; V- Ex vitro *adaptation or plant acclimatization*: where *in vitro* plants are adapted to the environment outside the laboratory conditions by managing the interaction of the plants and the "new" environmental conditions.

The first attempts to propagate *Prunus* species were successful during the 1960s (Martínez-Gomez et al., 2005) and, after that, economically important species were propagated using meristems (Perez-Tornero et al., 1999; Kalinina et al., 2007), axillary buds (Muna et al., 1999) or shoot tips (Sulusoglu and Cavusoglu, 2013).

Most of the investigation in *Prunus* species have focused on the optimization of the propagation step, since morphogenesis appears to be highly genotype-dependent. In apricot (*Prunus armeniaca* L.) cv. "Hacihaliloglu", it was found that by adding cytokinin 6-Benzyladenine (BA) (at either 2.0 mg L^{-1} or 1 mg L^{-1}) the propagation efficiency increased. The use of sucrose as a carbon source also improved shoot production. Rooting of the plants was best achieved on Murashige and Skoog (MS) medium supplemented with 2.0 mg L^{-1} indole-3-butyric acid (IBA) (Yildirim et al., 2011). However, a different pattern of morphogenic response was obtained for the cultivar 'Bebecou', where the combination of 2.2 μM BA and 0.57 μM IAA induced the best micropropagation rate (Koubouris et al., 2006). Especially in apricot, it was previously established that cultivars needed different media compositions to develop efficient shoot formation and plant development (Perez-Tornero and Burgos, 2000).

Micropropagation of plums has also been developed for different cultivars. Japanese plum (cv. "America") was successfully propagated under *in vitro* conditions by using young shoots. It was also found that cultivating the *in vitro* plants with 1.0 mg L^{-1} of IAA yielded the highest plant survival during the *ex vitro* step (Bandeira et al., 2012). For Japanese plums cv. "Gulf ruby" the micropropagation protocol was established from mature node explants and after that the explants were cultivated onto Woody Plant Medium supplemented with plant growth regulators for shoot induction and elongation. Plant survival after the *in vitro* stages was obtained under greenhouse conditions with a very high success rate (Zou, 2010).

In sweet cherry, the application of micropropagation techniques is widely exploited, especially for rapid propagation of new genotypes (Isac et al., 2010). Sweet cherry micropropagation from field shoots was found to be useful for eliminating phytoplasms, but not viroids (Staniene et al., 2009). Regeneration of adventitious shoots from leaves and, for the first time, from internode sections has been obtained in five important sweet cherry cultivars ("Schneiders", "Sweetheart", "Starking Hardy", "Giant", "Kordia" and "Regina"). Additionally, it was found that all the cultivars behaved better in the media DKW/WPM (1:1) and Quoirin/Lepoivre (QL) with the addition of thidiazuron in combination with indole-3-butyric-acid. Node explants produced more shoots than the leaf explants (Matt and Jehle, 2005). More recently, in cherry laurel (*Prunus laurocerasus* L.), a new species with high commercial potential, an efficient protocol for multiplication from embryos was established in plant regulator-free medium and the *ex vitro* plant developed into phenotypically normal plants (Sulusoglu, 2012; Sulusoglu and Cavusoglu, 2013).

B. Rootstock micropropagation and micrografting in Prunus sp.

Vegetative propagation of *Prunus* species traditionally occurs by cuttings or grafting. In the latter case, farmers use suitable rootstocks to confer resistance against several kinds of abiotic and biotic stresses, keeping the desired characteristics of the cultivar in the scion. In *Prunus*, rootstocks are propagated by seeds or by cuttings. Propagation by seeds can generate a large amount of genetic variation in the rootstocks, affecting grafting efficiency and plant yield in the field. On the other

hand, cuttings harvested from field plants can also reduce the efficiency of the grafting process and can spread diseases into the new plantations (Vujović et al., 2012). It is evident, for both the industry and breeders, that tissue culture has the potential to produce rootstocks on a large scale faster than the traditional methods. Additionally, this technology can guarantee the production of genetically identical, physiologically uniform and pathogen-free plants at reasonable costs (García-Gonzáles et al., 2010; Vujović et al., 2012).

Strenuous efforts have been made to propagate rootstocks of several *Prunus* species (Da Rocha et al., 2009) such as peach (Radmann et al., 2009), sweet cherry and plum (Vujović et al., 2012). As in many fruit species, setting and optimization of *in vitro* culture parameters have been necessary to develop reliable protocols and to extend these protocols to a large-scale commercial level. Variation among the different rootstock species (Krizan et al., 2007) as well as optimization of the different culture and media compositions, have been factors that it has been necessary to face in order to implement successful propagation of *Prunus* rootstocks (Fotopoulos and Sotiropoulus, 2004).

In vitro shoot tip grafting (STG) or micrografting is the aseptic grafting of a small shoot tip onto an *in vitro* rootstock produced by seeds or by asexual propagation (Navarro et al., 1975). This technology has been widely used for producing virus-free plants (Navarro et al., 2002) and has emerged as an alternative option to exchange *Prunus* genetic material among countries (Conejero et al., 2013). However, more effort needs to be made towards determining the genetic and physiological barriers at the tissue and/or cell level, so that this technique may be optimized and exploited to its full potential (Monteuuis, 2012).

C. Production of virus-free plants by tissue culture

Virus elimination in *Prunus* species has been achieved using different techniques. In peach and almond, disease free plants were produced for the viruses **Prunus Necrotic Ring Spot Virus** (PNRSV), **Prune Dwarf Virus** (PDV), A**pple Mosaic Virus** (ApMV) and **Apple Chlorotic Leaf Spot Virus** (ACLSV) using the STG technology, with better results than the use of thermotherapy (Navarro et al., 1982; Juárez et al., 1988; Juárez et al., 1992). Modifications of the STG protocols have been effective for eliminating recalcitrant viruses that were not eliminated by thermotherapy and/or chemotherapy of meristem culture (Conejero et al., 2013). In this case, the STG technology allows the micrografting of free shoot tips from diseased plants onto a selected rootstock, giving the possibility to produce the non-infected plant (Navarro et al., 2002).

However, thermotherapy was effective for the elimination of ACLSV in apricot, PNRSV and ACLSV in peach and PDV and ACLSV in sweet cherry. The effectiveness was very different for each kind of virus-host system; 37-100% of virus-free plants were obtained for PNRSV, 60-100% for ACLSV and 85-100% for PDV.

Meristem culture has also been useful for the elimination of the Plum Pox Virus (PPV) and PNRSV from infected nectarine shoots (cv. "Arm King") (Manganaris et al., 2003). In this case, the previous application of thermotherapy to potted trees for 3 weeks at 35 ºC improved the excision of longer meristems and this increased the regeneration response (38%) in woody plant medium (WPM) without plant growth regulators. The efficiency of virus elimination reached 86% for PPV and 81% for PNRSV detected by DAS-ELISA and multiplex RT-PCR, respectively.

Also, cryotherapy of shoots has appeared as a new and alternative method for pathogen eradication based on cryopreservation techniques (Wang et al., 2009). Cryopreservation is the maintenance of biological samples at ultralow temperature, using liquid nitrogen (-196 ºC). In cryotherapy, plant pathogens (viruses, phytoplasmas, virodis, micoplasmas and bacteria) are eliminated from shoot tips by a short exposure to liquid nitrogen. It is possible to enhance the effectiveness of thermotherapy by using cryotherapy of the target tissues before or after the thermotherapy treatments. In the interspecific *Prunus* rootstock Fereley-Jaspim, cryotherapy of shoot tips increased the elimination of the *Plum Pox Potyvirus* Marcus strain from 20% to 50%, compared to the use of meristem culture technique (Brison et al., 1997).

D. Haploid tree production by tissue culture

Haploids are sporophytic plants with the gametophytic chromosome number because they come from a single gamete. The exploitation of haploids or di-haploids (obtained by doubling the chromosome numbers or DH) has been extensive because the time needed to generate completely homozygous lines is shorter than with conventional breeding. Indeed, haplo-diploidization through gametic embryogenesis can produce homozygous lines from heterozygous parents in a single-step process. On the other hand, by conventional methods pure lines are developed over several generations of self-crossing, which still does not give complete homozygosity. This technology is very suitable in woody fruits because they have long reproductive cycles, high degrees of heterozygosity and self-incompatibility. The absence of pure lines in woody trees makes genetic studies rather difficult to conduct (Germaná, 2006).

In several cultivars of apricot it was possible to induce the formation of multicellular pollen and proembryos by managing the nutrient composition of the basal medium. The reprogramming of the microspore and the first steps of the embryogenic pathway were obtained, thus opening the possibilities for production of microspore-derived embryos and DH plants (Germaná et al., 2011). Before this study haploid calli induction from anthers was also obtained in apricot *cv.* Harcot, and the microspore origin of these calli was confirmed (Peixe et al., 2004).

Meanwhile, in sweet cherry production of haploid calli was achieved by using anther culture (Hofer and Hanke, 1990) but with no regeneration of plants.

E. Embryo rescue

The stone fruit breeding programs have been focused on the development of early ripening cultivars, with short fruit development periods (FDP) (Scorza and Sherman 1996). For most of the *Prunus* species with short PDF, one of the main constraints affecting the germination of hybrid progenies is embryo abortion. In these case, fruit ripening occurs before the embryo can complete its morphological and physiological development. In consequence, the embryo cannot uptake key metabolites and plant growth regulators to complete the embryogenesis and to germinate. Embryo rescue technology

has been applied for germination of plants originated from early maternal lines. Embryo rescue consists of the aseptic cultivation of immature -or even mature- embryos in a plant tissue culture medium. The basal medium can be supplemented with plant growth regulators, vitamins and amino acids to complete the physiological maturation of the embryo and to induce plant germination (Hartmann et al. 1997). Embryo rescue has also been used to develop interspecific plant hybrids in which the embryo normally would degenerate and would not be able to germinate. In *Prunus* this technique allows crossing between two early ripening cultivars or, more specifically, the use of early ripening genotypes as female progenitors (Hartmann et al. 1997; Pinto et al. 1994).

For embryos harvested during the last stage of embryogenesis the nutrient requirements in the medium to germinate are fewer than for those embryos collected at early stages of development (Scorza and Sherman, 1996). MS (Murashige and Skoog, 1962); basal salts and vitamins as well as Woody Plant Medium (WPM) (Lloyd and McCown, 1981) with several modifications are the most widely used tissue culture media for embryo rescue.

Finally, it is important to mention that success of embryo rescue depends on the species, parental genotypes, culture media, stratification period and plant growth regulators (Emershad and Ramming, 1994; Jeengool and Boonprakob, 2004).

III. GENETIC TRANSFORMATION

Genetic transformation of crop plants has been successfully applied to breeding programs in order to express some traits difficult to acquire by conventional breeding. In woody species such as the genus *Prunus* genetic transformation is a powerful tool, because traditional breeding has several limitations such as long juvenility, genotype autoincompatibility and heterozygosity (Petri and Burgos, 2005).

In vitro shoot regeneration efficiency has been the main problem in genetic transformation protocols in fruit tree species. Factors affecting cell induction for shoot regeneration include the explant type, media components, growth regulators and environmental factors such as light, temperature and photoperiod, and may be different for each genotype (George et al., 2008). Selection of the appropriate *Rhizobium radiobacter* strain (syn. *Agrobacteruim tumefaciens*), infection conditions and selection markers also affect the efficiency of the transformation process (Petri and Burgos, 2005).

Genetic transformation in European plum (*Prunus domestica* L.) has been established through regeneration of the cotyledon and hypocotyl sections of mature embryos (Mante et al., 1989; Scorza et al., 1994), immature embryos and embryonic axes (Tian et al., 2007a; Srinivasan et al., 2012; Petri et al., 2012: Wang et al., 2013). Medium supplemented with thidiazuron (TDZ) 7.5 μM in combination with indole-3-butyric acid (IBA) 0.25 μM has been used for shoot regeneration, with a transformation efficiency of 1.2% (Scorza et al., 1994; González-Padilla et al., 2003). Petri et al. (2012) reported 42% transformation efficiency increase using 9 μM 2,4-dichlorophenoxyacetic acid (2,4-D) for three days during co-cultivation, followed by a regeneration medium containing TDZ (7.5 μM) and kanamycin 80 mg L-1 as selective agent. Using this protocol, Srinivasan et al. (2012) successfully transformed European plum with the *Pt*FT1 gene.

Wang et al. (2013) first reported the selection and regeneration of transgenic plants with the selection marker from *E. coli*. phosphomannose isomerase (*pmi*). The use of this marker gene is relevant because of its lower environmental risk for commercial release.

A European plum resistant to plum pox virus (PPV) "Honey Sweet" (developed from the C5 transgenic line) was the first transgenic commercial *Prunus* released in which the virus resistance trait is heritable and stable. Virus resistance is due to the phenomenon of post- transcriptional gene silencing (PTG) given by the insertion of a transgene-multicopy complex and aberrant copies of the virus coat protein (PPV-CP) (Scorza et al., 1994; Ravelonandro et al., 2000; Scorza et al., 2001).

The stability of 'C5' has been evaluated under field conditions in different countries, showing that it can spend several years without becoming infected (Scorza et al., 2001). Also confined trials have been performed using C5 micro-grafted on the rootstock Adesoto 101 (*Prunus insititia* L.) previously infected with PPV local isolates. Results showed that after three years of growth in a greenhouse some plants showed mild symptoms on leaves, suggesting the importance of long-term trials (Wong et al., 2010).

European plum plants with high expression of the flowering locus T1 (FT1) from *Populus trichocarpa* showed a continuous reproductive phenotype (flower and fruit) under greenhouse conditions, and during spring and autumn in the field (Srinivasan et al., 2012).

In Japanese plum, adventitious shoot regeneration from hypocotyls and cotyledons of mature seeds has been reported (Tian et al., 2007b; Urtubia et al., 2008; Canli and Tian, 2009). Shoots were induced from hypocotyl segments of mature seeds on medium containing TDZ, 6-benzylaminopurine (BA) and kinetin. TDZ medium was the most effective for shoot regeneration, with an efficiency of 30% of explants forming shoots; the shoot induction response was genotype-dependent (Tian et al., 2007a).

Urtubia et al. (2008), using a modified protocol for regeneration of European plum (Scorza et al., 1994), obtained 11% and 19% shoot regeneration from hypocotyl slices in the Japanese plum cultivars "Angeleno" and "Larry Anne", respectively. The best results for "Angeleno" were obtained using TDZ at 3 or 5 μM in combination with IBA 0.5 μM and 5 or 7 μM TDZ in combination with IBA 1 μM. For 'Larry Anne' the best treatment was TDZ 3 μM combined with IBA 0.5 μM and TDZ 7 μM in combination with IBA 1 μM. An average transformation efficiency of 0.6 was obtained with *R. radiobacter* strain GV3101 harboring a plasmid with neomycin phosphotransferase (*npt*II) and the green fluorescent protein (GFP).

Responses to different TDZ concentrations were evaluated in mature cotyledons for shoot regeneration in five cultivars of Japanese plum. TDZ responses were strongly genotype-dependent between 3.75 and 15 μM in QL medium (Quorin and Lepoivre, 1977). Incubation under dark conditions significantly increased shoot induction. Shoot regeneration varied among genotypes from 6.7% to 66.7% (Canli and Tian, 2009).

In peach, genetic transformation has been difficult to achieve because of the high recalcitrance to regeneration. Shoot regeneration has been reported from the proximal region of cotyledons from immature embryos, hypocotyl segments, longitudinal sections of mature embryos (Mante et al., 1989; Pérez-Clemente et al., 2004) and leaves from shoots cultured *in vitro* (Gentile et al., 2002).

Transformation in peach has been reported using *Rhizobium*-mediated transformation of immature embryos (Gutiérrez-Pesce et al., 1998, 2004; Petri et al., 2012). However, the regeneration of plants from transgenic tissues is still difficult and the recovery of transformed plants has been difficult to date.

An efficiency of 3.6% was obtained in peach transformation using the *R. radiobacter* strain C58 and regenerating the plants in medium supplemented with 7.5 μM TDZ and 2.4 μM indolacetic acid (IAA) (Pérez-Clemente et al., 2004). Studies conducted by Padilla et al. (2006) evaluated various explants, *R. radiobacter* strains, different vectors and promoters; the best transformation rate (56.8 %) was obtained using epicotyl internodes.

Genetic transformation in cherry has been reported for a few commercial genotypes such as sour cherry, black cherry (*Prunus serotina* Ehrh), chokecherry (*Prunus virginiana* L.) and rootstocks such as "Gisela 6" and "Gisela 7" (*P. cerasus* x *P. canescens*), "Colt" (*P. avium* x *P. pseudocerasus*), "Rosa" (*Prunus subhirtella autumno rosa*), "Inmil" (*P. incisa* x *P. serrula*) and "Damil" (*P . dawyckensis*) (Da Cámara Machado et al., 1995; Druart et al., 1998, Gutierrez-Pesce and Rugini, 2004; Gutierrez-Pesce et al., 1998; Liu and Pijut, 2010; Song and Sink, 2005, 2006; Song et al, 2013).

Transformed plants of "Colt" cherry rootstock (*P. avium* x *P. pseudocerasus*) containing the genes *rol A*, *B* and *C* of the non-disarmed *A. rhizogenes* pRi1855 TDNA were obtained with enhanced rooting capacity, shortened internodes and wrinkled leaves phenotype (Gutiérrez-Pesce et al., 1998, 2004; Dolgov and Firsov, 1999). Also, transformation in "Gisela 6" and "Gisela 72" cherry rootstocks with resistance to *Prunus* necrotic ringspot virus (PNRV) through RNAi-mediated silencing was recently reported. This represents an interesting application that indirectly could solve the problems associated with this disease in cherry varieties for fruit production (Song et al., 2013).

Regeneration and transformation protocols for *P. cerasus* (Mante et al., 1989; Tang et al., 2002; Song and Sink, 2005, 2006) and *P. avium* (Hammatt and Grant, 1998; Tang et al., 2002; Matt and Jehle, 2005; Feeney et al., 2007) were based on adventitious regeneration using leaves and internodes from plants grown *in vitro* and hypocotyl slices.

In sour cherry cv. "Montmorency", a pretreatment in MS (Murashige and Skoog, 1962) liquid medium with a low concentration of TDZ (0.45 μM) for 24 h followed by culture on shoot regeneration medium with BA 13 μM and naphthalenacetic acid (NAA) 2.7 μM improved the regeneration frequency compared to the non-TDZ treatments (Song and Sink, 2005). Stable transformation with 3.1% efficiency was achieved in cv. "Montmorency" using BA 0.5 mg l–1 and IBA 0.05 mg L^{-1} in QL medium (Song and Sink, 2006).

Genetic transformation technology in *Prunus* fruit species is still at a basic stage of development. Further research is needed in the different species and varieties to develop an efficient and high performance transformation system.

IV. GENOME SEQUENCING AND TRANSCRIPTOME INITIATIVES IN PRUNUS: THE SEARCH FOR CANDIDATE GENES AND SNPS FOR MARKER ASSISTED BREEDING PROGRAMS

Over the past decade several International Consortia have been working towards deciphering the genomes of several members of the Rosaceae, uniting scientists from Belgium, Chile, France, Germany, Israel, Italy, New Zealand, Norway, Spain, South Africa, UK, and USA; these international consortia have published draft genomes of peach, apple and strawberry (Velasco et al., 2010; Shulaev et al., 2011; Verde et al., 2013). Additionally, the genomes of sweet cherry, pear and almond have recently been made publically available (http://genomicsdata.wsu.edu/public_access/index.php).

These genome initiatives coupled with transcriptomic analyses of these fruit species are enabling scientists to identify candidate genes, signal transduction pathways and metabolic pathways that may play an important role in fruit quality and production (Gonzalez-Aguero et al., 2008; Tittarelli et al., 2009; Vizoso et al., 2009; Illa et al., 2011a; Alkio et al., 2012; Barakat et al., 2012; Diez-de-Medina and Silva, 2012; Dirlewanger et al., 2012; Habu et al., 2012; Leida et al., 2012; Trainotti et al., 2012; Koepke et al., 2013; Eduardo et al., 2013; Martinez-Garcia et al., 2013; Sanchez et al., 2013). Identification of polymorphisms between members of the Rosaceae, members of the same genus (i.e. *Prunus*) or within different varieties/cultivars of a particular species is providing correlative evidence of potential polymorphisms that may be associated with quality traits (Fernandez et al., 2012; Martínez-García et al., 2013; Koepke et al., 2013). Association of these quality traits with the polymorphisms in segregating mapping populations may provide direct evidence of the role that these polymorphisms play in phenotypic variation. Recent genomic advances are giving rise to technological platforms that enable researchers to identify large numbers of polymorphisms and create of high density linkage maps (Klagges et al., 2013). Association of these molecular markers with quantitative trait loci (QTLs) has the potential to provide useful and cost effective tools for marker assisted breeding programs (Dirlewanger et al., 2012; Eduardo et al., 2013).

A. Conserved molecular markers among members of the Rosaceae

Genomic co-linearity and marker transferability has been demonstrated within members of the Rosaceae (Joobeur et al., 1998; Dirlewanger et al., 2004a, 2004b; Lambert et al., 2004; Dondini et al., 2007; Olmstead et al., 2008; Clarke et al., 2009; Cabrera et al., 2009). In 2009, a set of onserved Rosaceae gene-based sequences corresponding to single copy *Arabidopsis* genes was developed (Cabrera et al., 2009). These molecular markers, RosCOS (Rosaceae Conserved Orthologous Set), were constructed using the available Rosaceae ESTs from *Malus*, *Prunus* and *Fragaria*. These analyses led to the development of 613 RosCOS markers that were mapped to the *Prunus* TxE reference map (almond "Texas" x peach "Earlygold"), resulting in a genome-wide coverage of 0.67 to 1.06 gene-based markers per cM per linkage group (a total of eight linkage groups) (Cabrera et al., 2009). Comparative analyses of the map position of RosCos markers on the *Prunus* TxE reference map with their positions in the *Prunus, F. vesca* and *Malus* genomes has revealed macro-syntenic relationships between the *Prunus, Fragaria* and *Malus* genomes (Shulaev et al., 2011; Illa et al., 2011a, 2011b), suggesting that there is conservation in genome structure and function, and also that molecular markers and QTLs may be conserved among members of the Rosaceae.

B. Genome Database for Rosaceae (GDR)

In order to assist the international community in optimizing the wealth of information that is being generated towards

improved breeding programs an integrated web-based relational database was developed, the Genome Database for Rosaceae (GDR) (http://www.rosaceae.org) (Jung et al., 2008; Jung et al., 2004). The GDR database stores data associated with the genetically anchored peach physical map, annotated EST databases of apple, peach, almond, cherry, rose, raspberry and strawberry, Rosaceae maps and markers associated with a Comparative map viewer (CMAP), QTLs and MTLs (Mendelian Trait Loci), as well as Breeders toolbox of phenotypic and genotypic data.

C. Functional Genomic studies and Genome sequencing initiatives

Peach

One of the major problems for breeding programs is that only a limited number of genetic markers have been available. In 2012, an international consortium (The International Peach SNP Consortium; IPSC) performed a genome-scale SNP discovery in peach using next generation sequencing platforms to develop and characterize a high-throughput Illumina Infinium SNP genotyping array platform. A whole genome re-sequencing was performed on 56 peach breeding accessions using the Illumina and Roche/454 sequencing technologies. A set of 8,144 SNPs that were included on the IPSC peach SNP array v1, distributed over all eight peach chromosomes with an average spacing of 26.7 kb between SNPs was released (Verde et al., 2012). Additionally, the peach genome has been sequenced (Verde et al., 2013). The work describes the high-quality whole-genome shotgun assembly of a double haploid genotype of the peach cv. "Lovell" (PLov2-2N; 2*n* = 2x = 16) with an estimated genome size of 265 Mb. 27,852 protein-coding genes were predicted, as well as noncoding RNAs. The information available in this genome is enabling scientists to perform comparative genomic analysis between different species. Based upon what has been seen in terms of the macro-synteny at the genomic level of members of the Rosaceae, these analyses should facilitate the identification and characterization of conserved metabolic pathways as well as the discovery of molecular markers associated with quality traits. A comparative analysis of three *Prunus* species (peach, almond and sweet cherry) by Koepke et al. (2013) has revealed the potential information that may be obtained by performing comparative genomic analyses between different species. Using the peach genome as a reference genome, sequencing reads from four almond accessions and one sweet cherry cultivar were compared for polymorphisms in candidate genes. In these analyses, the reference mapping enabled the identification of many biologically relevant species-specific polymorphisms. These species-specific polymorphisms may be the cause of the phenotypic variation detected in the different species. Specifically, comparing peach with sweet cherry nonsense SNPs were detected in two 1-aminocyclopropane-1-carboxylate synthase (ACS) genes and two 1-aminocyclopropane-1-carboxylate oxidase (ACO) genes in sweet cherry. Since sweet cherries are non-climacteric and peaches are climacteric, these polymorphisms may be the origin of the difference between climacteric and non-climacteric fruits (Koepke et al., 2013). Comparative analyses between sweet and bitter almonds also revealed a set of candidate genes with nonsense mutations that were polymorphic between these two types of almonds, possibly causing the phenotypic differences between these two varieties. This work is the first report in plants that associates nonsense SNP abundance in a genus with specific GO terms and potential phenotypic variation.

Sweet cherry

The sweet cherry genome and transcriptome sequencing information have provided new opportunities to study the expression and structure of genes involved in abiotic stresses such as cracking, with the aim to develop new tolerant cultivars (Balbontin et al., 2013). By performing RNA-seq and qPCR analyses on sweet cherry cultivars with different susceptibility to cracking, Silva et al. (2012) identified differential expression of genes associated with alkene synthesis, suggesting that the differential expression of these genes may be associated with differential susceptibility to cracking.

A research team at Institut National de la Recherche Agronomique INRA-Bordeaux (France) has also recently initiated a research program using a classical quantitative trait locus approach to identify the genetic basis of fruit cracking tolerance (Balbontin et al., 2013). A genetic map was developed based on 125 full-sib hybrids of a cross between "Regina" (one of the most cracking-tolerant commercial cultivars) and "Lapins" (having intermediate tolerance to cracking). Recently, high density sweet cherry linkage maps have been developed with high saturation of Single Nucleotide Polymorphism (SNP) markers (Klagges et al., 2013), using the RosBREED cherry 6K SNP array v1 (Peace et al., 2012). The sequencing of the sweet cherry genome was also initiated a few years ago (Koepke et al., 2010).

During 2013, the genome sequence has been made publicly available with an actual coverage of 83.5x (http://genomicsdata.wsu.edu/public_access/index.php, Table 3) (Dhingra and Silva, unpublished results). The availability of the sweet cherry genome and comparative analyses with the genome sequence of other *Prunus* and Rosaceaes are providing the information necessary to identify genes, signal transduction pathways and putative markers associated with disorders such as cracking.

TABLE 3

Sequencing data of the sweet cherry genome

Data Type	Amount	Coverage (x)	Genome Size
454 – single	1 Gb	4.44	~225 Mb
454-8 kb Paired	63.7 Mb	.28	
454-20 kb paired	116.5 Mb	.52	
Illumina (56 bp)	556.0 Mb	2.47	
Illumina (100 bp)	17.2 Gb	76.4	
PacBio	21.2 Mb	.09	
Total	**18.78 Gb**	**83.5**	

The data has been provided by the Sweet cherry International Consortium headed by Amit Dhingra (Washington State University, USA) and Herman Silva (Universidad de Chile, Chile).
See, http://genomicsdata.wsu.edu/public_access/index.php

Plum

Recently, efforts are also underway to understand the plum transcriptome. Transcriptomic analyses are underway to identify differentially expressed genes in plum varieties with different phenotypes such as color, anthocyanin content and flavor (González M, Carrasco B and Silva H, unpublished results). Since there is macrosynteny among the members of the Rosaceae family and within the genus *Prunus*, the peach and sweet cherry genomes will serve as reference genome to map the transcripts in these studies. Presently, there is no initiative to sequence the genome of *Prunus salicina*. However, work in the USDA ARS has begun to sequence the genome of several different cultivars of *Prunus domestica* (Dardick et al., 2011).

Perspective

Despite the efforts in recent years, our knowledge of fruit development and physiology at the genetic and molecular level is poor and not fully understood. Future studies towards understanding stone fruit physiology at a molecular level, as well as high throughput methods to quickly and efficiently cultivate and transform these species *in vitro* are needed. Mass propagation of new cultivars and rootstocks could be supported by the development of more efficient technologies such as temporary immersion system-based protocols, which are currently poorly investigated in these species. Additionally, further work is needed on regeneration of viable plants from haploid or di-haploid *in vitro* induced tissues. Without a doubt the information generated through the use of functional genomics tools as well as genome sequencing will yield a better understanding of how genes are regulated, how genes interact with others and how they can be used in breeding programs assisted by molecular markers, in the near future.

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