Biotechnological Advances in the Genetic Improvement of *Prunus domestica*

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Abstract

Plum producers world-wide are facing multiple challenges including climate change, reductions in available labor, the need for reduced chemical inputs, the spread of native and exotic pests and pathogens, and consumer demands for improved fruit quality and health benefits. Meeting these challenges will require innovation in many areas of science and technology and especially in plum breeding. In an effort to develop new approaches to plum improvement the USDA-ARS Appalachian Fruit Research Station fruit breeding program in collaboration with partners in the U.S. and Europe have developed a genetic engineering (GE) approach to target resistance to *Plum pox virus* (PPV) the causal agent of sharka, one of the most destructive diseases of plum. This program has resulted in the development of a GE plum cultivar 'HoneySweet' which has been tested for 15 years in the European Union and in the U.S. and is highly resistant to PPV. 'HoneySweet' has received full regulatory approval in the U.S. 'HoneySweet' represents a new source of PPV resistance for growers and it can be used by breeders to develop additional resistant cultivars and/or rootstocks.

Rapidly incorporating important traits into improved plum cultivars requires new approaches to breeding that can reduce or eliminate breeding limitations such as long juvenility periods; the need for extensive and costly breeding plots; and yearly limitations on flowering and fruiting related to seasonal dormancy. To address these limitations the USDA group and partners in the U.S. have developed a system to shorten the breeding cycle of plum. We have overcome the juvenility and environmental limitations of flowering and fruiting by incorporating a gene that induces trees to flower early and continually. We have reduced the plum generation cycle from 3-7 years to less than one year. We call this rapid breeding system "FasTrack". The system allows for the rapid incorporation of important traits into plums and then in the final generation, when substantial improvements are clearly evident, only seedlings that do not contain the early flowering transgene are selected. The selected trees may then be used directly as new cultivars, or improved lines for further breeding. Genetic engineering of important traits, FasTrack breeding, and other approaches that are under development will allow the latest advances in biology to be applied to improving and sustaining plum production.

INTRODUCTION

Plums (*Prunus domestica* L.) are a good source of dietary fiber, sorbitol, potassium, copper, boron, and phenolic compounds which are active in health promoting functions. Dried plums and prune juice are known for their supportive role in normal digestive functioning (Somogyi, 2005) and preventing age-related bone loss (Armandi et al., 2001, 2002; Bu et al., 2007; Deyhim et al., 2005; Franklin et al., 2006; Halloran et al., 2010). California is the world leader in dried plum production providing 99% of the U.S. supply and 40-60% of the world supply. In 2011 California exported over 68,000 metric

tons of dried plums to 44 countries (http://www.californiadriedplums.org/media/ce053413/2010-1%20Final%20Statistical%20Report.pdf). 'Improved French' (a.k.a. 'French') which was brought to California in 1856 represents 98% of the total dried plum acreage in California. This monoculture situation lends itself to vulnerability to disease and pest outbreaks and statewide yield fluctuations due to the effects of weather that can negatively effect fruit set and/or fruit retention. In addition to the risks of monoculture, due to the preponderance of a single cultivar, the entire industry must harvest and dehydrate the crop within the span of a few weeks. The development of new dried plum cultivars that conform to, or exceed, industry standards will increase the efficiency of California dried plum production and give some protection against the risks involved with a monoculture. The industry has identified breeding priorities for new cultivars that focus on tolerance to pests and diseases, improved consumer traits such as improved flavor and nutritive value, and cost saving characteristics such as lower drying costs and reduced pruning. High sugar levels are a major need identified by the industry in terms of increased consumer preference and due to the fact that higher sugar levels decrease the drying time. The drying process is a major cost of dried plum production.

The University of California, Davis Dried Plum Development and Evaluation Program (T. DeJong) was initiated in 1985 as a continuation of a program initiated in 1975. This breeding program serves the needs of the California dried plum industry through the development of new cultivars. Two cultivars – 'Sutter' and 'Muir Beauty' have been released but the dried plum industry remains overwhelmingly dependent upon a single cultivar, 'Improved French'. One of the particular vulnerabilities of this cultivar is its susceptibility to *Plum pox virus* (PPV) (Scorza and Damsteegt, pers. commun.).

PPV is the most destructive disease of plums and other stone fruit species. First reported from Bulgaria at the beginning of the 20th century, it spread rapidly throughout Europe and later in the century was reported in Asia, Africa, and North and South America (see Various Authors, 2006). World-wide, it is estimated that PPV has caused $13 billion in losses over the last 30 years, and current losses in Europe amount to $180 million each year (Cambra et al., 2006). The U.S. plum industry is under threat from PPV. In 1999 PPV was detected in Pennsylvania. Over $40 million was spent over a ten year period in Pennsylvania to eradicate the disease. In the summer of 2006, a national surveillance program detected PPV outbreaks in New York and Michigan. Although federal and state authorities are working to prevent disease spread through quarantine and eradication programs, the detection of PPV in two additional states (PPV was found in one tree and was eliminated from Michigan) clearly indicates that U.S. growers remain at risk from future PPV outbreaks. Canada maintained a PPV eradication program for over 10 years but has recently announced plans to monitor and manage, rather than eliminate the virus (http://www.inspection.gc.ca/plants/plant-protection/diseases/plum-pox-virus/monitoring-and-management-program/eng/1323887724804/1323889930176). For the stone-fruit industry (plum, peach, apricot, cherry, almond), it may only be a matter of time before we see a continued spread or a re-introduction of PPV in the United States, given the current presence of the disease in North America (New York and Canada) and in South America (Chile and Argentina). There is no effective conventional method for controlling PPV infection. Rarely has PPV been eradicated from a country. The US industry is at serious risk.

PPV eradication programs would be potentially catastrophic for the California industry. In Pennsylvania the PPV eradication effort led to a near elimination of stone fruit production in the impacted counties. PPV resistant plum cultivars would provide the industry with a long-term, sustainable solution to this disease threat. Yet, there are few sources of resistance naturally occurring in *P. domestica* germplasm. Resistance based on hypersensitivity shows some promise but this resistance is multigenic, may not have long-term efficacy, and would take many years to transfer to new cultivars due to its multigenic nature (Hartmann, 2004; Neumüller et al., 2010). The industry may not have the luxury of time. Beyond hypersensitivity, there are no well-documented sources of

resistance. Many reports of resistance appear to be tolerance (symptomless infected trees) which is not desirable in controlling the spread of PPV.

To provide timely and effective solutions to the problems of PPV vulnerability and other threats, both biotic and abiotic, researchers at the United States Department of Agriculture, Agricultural Research Service Appalachian Fruit Research Station in Kearneysville, West Virginia, along with US and international colleagues are building plum genetic improvement biotechnologies and infrastructure that include genetic engineering and accelerated breeding.

Genetically Engineered PPV Resistance

In 1992 the PPV coat protein (CP) gene was isolated and sequenced (Ravelonandro et al., 1992). In collaboration with Ravelonandro, Dennis Gonsalves, and members of Gonsalves' research group, the PPV-CP gene was engineered into the plasmid pGA482GG (Fitch et al., 1990; Ling et al., 1991), the same plasmid that was used for the successful engineering of *Papaya ringspot virus* resistant papayas (Fitch et al., 1992). *Agrobacterium*-mediated transformation of plum was based on the procedure developed by Mante et al. (1991) (improved by Petri et al., 2008) utilizing hypocotyl slices from seed derived from open pollination. Transgenic plants were transferred under a USDA-Animal and Plant Health Inspection Service (APHIS) permit to the BSL3-P containment greenhouse at the USDA-ARS Foreign Disease and Weed Research Unit at Ft. Detrick, MD. During the 3 years of these greenhouse-based inoculation and testing studies, the transgenic plum line C5 appeared to be highly resistant to PPV. However, this line did not express PPV-CP and produced barely detectable levels of CP mRNA. Clones that did express the CP gene proved to be susceptible (Ravelonandro et al., 1997; Scorza et al., 2001). This suggested that a mechanism other than CP-mediated protection was functioning. The C5 plum clone became the focus of research on the mechanism of resistance to PPV which was demonstrated to be based on post-transcriptional gene silencing (PTGS) (Ravelonandro et al., 1997; Scorza et al., 2001; Hily et al., 2004, 2005). Silencing was based on the activity of a hairpin configuration that was apparently the result of a duplication and rearrangement during the insertion event. While the C5 clone appeared to be highly resistant in greenhouse tests, field testing under artificial inoculation and natural aphid-vectored disease pressure was necessary to evaluate resistance on mature trees under typical orchard conditions, in different plum-growing environments, and with different PPV strains. In 1996 collaborations were developed with research partners in Europe (T. Malinowski, Poland; I. Zagrai, Romania; M. Cambra, Spain, and in 2002 with J. Polak in the Czech Republic) to test this resistant clone in areas where PPV was established. Appropriate field test permits were granted in each country. By 2002 the field tests clearly demonstrated the resistance of C5 to PPV infection through aphid vectors and by graft inoculation (Hily et al., 2004). Continuation of these tests through 2005 confirmed the resistance (Malinowski et al., 2006). C5, later patented as 'HoneySweet' (http://www.freepatentsonline.com/PP15154.html) remained PPV-free during >15 years of field testing in PPV infested regions of Europe. This cultivar, after 7 years of regulatory scrutiny, was approved by all of the pertinent US regulatory agencies (Animal Plant Health Inspection Service (APHIS), The Food and Drug Administration (FDA), and the Environmental Protection Agency (EPA)) (Scorza et al., 2012).

The development of 'HoneySweet' demonstrates the effectiveness of genetic engineering to produce a strong and stable form of resistance. The regulatory approval of 'HoneySweet' in the US demonstrates the safety and wholesomeness of this plum cultivar. 'HoneySweet' is a genetic resource with a highly effective and stable resistance to PPV. 'HoneySweet' can be grown as a resistant cultivar or used as a parent to develop new resistant cultivars. We have shown that 'HoneySweet' transfers the resistance trait to its progeny as a single dominant factor (Ravelonandro et al., 1998; Scorza et al., 1998). While 'HoneySweet' has been evaluated for fruit quality in the Czech Republic, Poland, Romania, and Spain, and in the Eastern U.S., it has not been tested in California and it has not been tested as a dried product. Developed from germplasm adapted to the eastern US

growing conditions ('HoneySweet' = 'Bluebyrd' open-pollinated (Scorza and Fogle, 1999)), 'HoneySweet' may not be suited to the growing conditions in California nor is it highly likely that it will have the combination of traits that are required for a dried product. 'HoneySweet' can be used as a parent in breeding new PPV resistant cultivars that are adapted to California and suitable for the drying industry. Nevertheless, we must contend with the slow pace of traditional plum breeding where each generation may require 3 to 6 years to reach sexual maturity.

Accelerated Breeding

The reduction or elimination of the non-reproductive juvenile stage of plum trees would provide the necessary breakthrough to speed the breeding of plum cultivars with resistance to PPV or with any other improved trait(s). The extreme reduction of juvenility has been achieved through the manipulation of genes involved in flowering, primarily transcription factors (Flachowsky et al., 2007). Transgenic early flowering apple trees have been shown to produce fruit with generation cycles of one year versus the typical 4-10 year non-flowering juvenile period (Flachowsky et al., 2007, 2009, 2011). The development of early flowering transgenic trees has been reported in several other tree species including citrus (Peña et al., 2001), poplar (Hoenicka et al., 2008), and pear (Matsuda et al., 2009) but in these species early flowering has generally not been translated into a systematic approach to breeding. In contrast, work with apple demonstrated the potential for developing breeding programs using early flowering transgenic lines (Flachowsky et al., 2011; LeRoux et al., 2011).

We have developed transgenic early flowering plums using the *Flowering Locus T1* (*FT1*) gene from *Populus trichocarpa*. Transgenic plums expressing this gene under the control of the *35S* promoter flower and produce fruit within one year following transformation and continue to flower and fruit when cultivated under greenhouse conditions (Fig. 1). Transgenic *FT1* plums in the greenhouse exhibit a highly branched shrub-like habit with weak, trailing lateral branches. They do not require a period of cold dormancy for flowering and are daylength insensitive (Srinivasan et al., 2012). Seedlings produced from hybridizations between non-transgenic and *FT1* plums segregate to early flowering shrubby *FT1*-expressing seedlings and non-transgenic seedlings that exhibit typical plum seedling growth and do not flower early.

In order to rapidly integrate the PPV resistance from 'HoneySweet' into germplasm adapted to the California plum industry 'HoneySweet' pollen was applied to *FT1* trees in the greenhouse. Progeny from these crosses segregate into four categories 1) non-*FT1* without the 'HoneySweet' PPV resistance insert, 2) non-*FT1* with the 'HoneySweet' insert, 3) *FT1* without the 'HoneySweet' insert, and 4) *FT1* with the 'HoneySweet' insert. Of these lines only those in class 4 are of interest. These *FT1*/'HoneySweet' seedlings have been hybridized with pollen from 'Improved French' and elite selections from the UC plum breeding program. We have also hybridized *FT1* plants with 'Improved French' and UC elite germplasm, and the *FT1* seedlings from these crosses have been hybridized with 'HoneySweet'. Genome spanning markers have been developed both from 'Improved French' and 'HoneySweet' and these markers, along with phenotyping will be used to select those seedlings that combine the 'HoneySweet' PPV resistance insert with traits necessary for the needs of the California industry. This approach promises to dramatically speed the backcrossing process providing one generation/year in place of one generation/3-6 years.

This program provides a single locus, dominant and highly effective PPV resistance from 'HoneySweet', accelerated modified backcross breeding with yearly generation cycles, and molecular markers to increase the efficiency of selection. Promising seedlings will have the same regulatory approvals as 'HoneySweet' and will not require further regulatory evaluation.

While the application of biotechnology to solve the problems that plum growers currently face and will face in the future is still at an early stage, genetic engineering, pathogen-derived resistance and accelerated breeding are providing approaches that can

provide significant benefits to growers and to consumers as we seek stable and efficient production and safe and healthful supplies of plums and other fruit crops.

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Figures

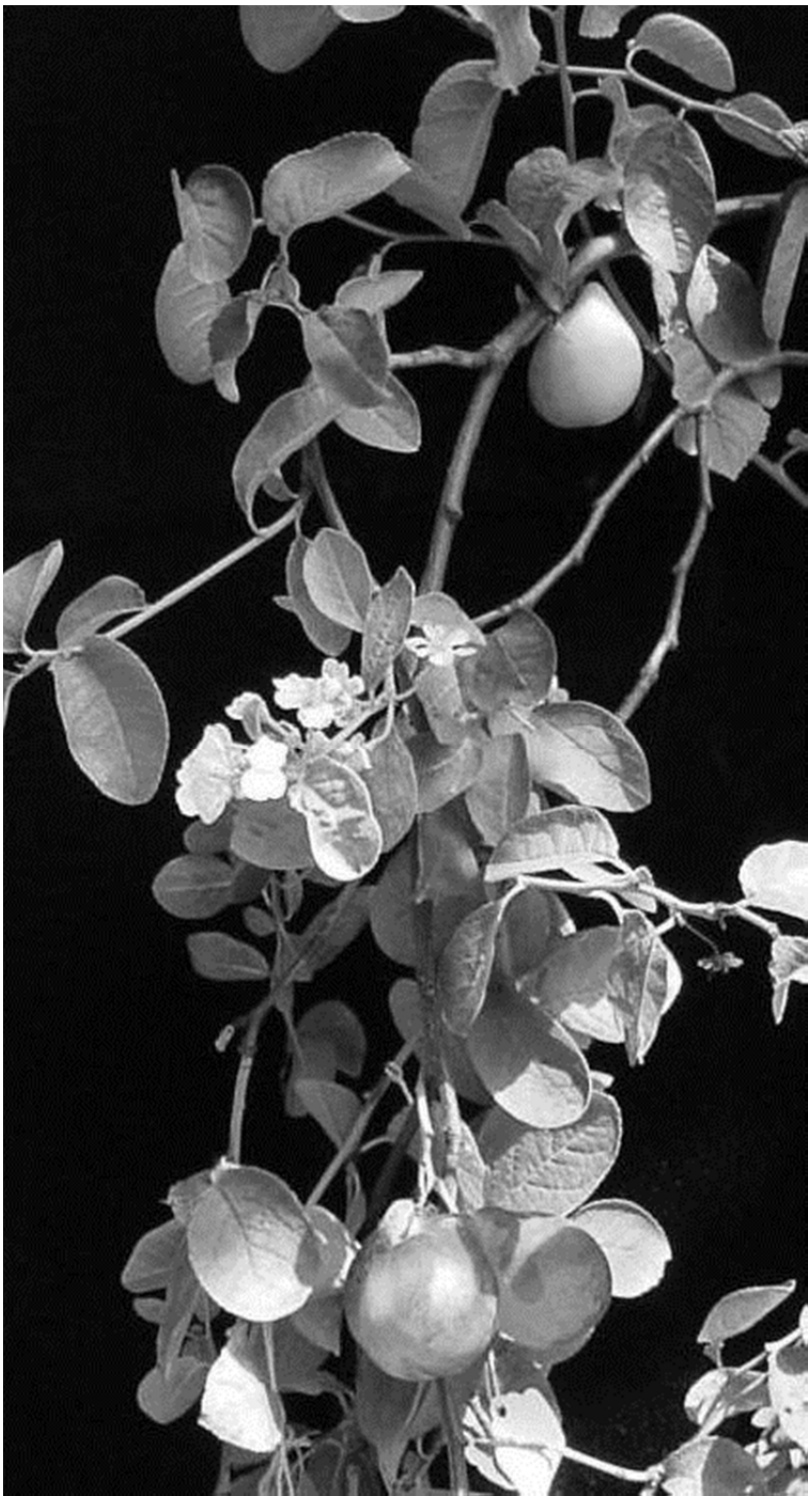

Fig. 1. Early flowering greenhouse-grown plum (*Prunus domestica*) plum with developing fruit and flowers.