RESEARCH RESULTS FOR THE YEAR 2008 'DEVELOPMENT OF PLUM POX VIRUS RESISTANT 'FRENCH PRUNE' ('IMPROVED FRENCH') PLUM.

Ralph Scorza
USDA-ARS Appalachian Fruit Research Station
Kearnsysville, WV 25443

OBJECTIVE

The objectives of this project are to 1) to develop genetically engineered clones of 'French Prune' that are highly resistant to PPV and 2) determine the level of susceptibility of 'French Prune' to PPV.

PROPOSED RESEARCH AND DEVELOPMENT

Since 1990, the USDA-AFRS Genetic Improvement Research Unit has been developing genetically engineered (GE) plum lines with different novel traits including resistance to PPV. 'HoneySweet' a GE plum highly resistant to PPV, developed in our laboratory, is undergoing the deregulation process in the U.S. We can consistently produce PPV resistant GE plums and have them in the greenhouse to test within 6 months. We propose to adapt this highly efficient transformation system to clonal material of 'French Prune'. Explants used to test regeneration efficiency will include leaves, petioles, internodes and roots. We have successfully developed GE pears and apples from clonal material and recently, a system for transformation of clonal European plum was reported for the Russian plum variety 'Startovaya'.

'French Prune' will be genetically engineered with a piece of a PPV gene to induce a natural plant resistance mechanism called "gene silencing" to specifically destroy PPV. A number of lines will be tested in the containment greenhouse for resistance. Molecular tests will be carried out to confirm that the PPV resistance mechanism is active and stable.

Based on our experience with previous transgenic plum lines we will utilize gene constructs with minimal intellectual property issues and constructs with the highest potential for consumer acceptability. Gene constructs will utilize only plant gene sequences (promoters and terminators). We will also investigate the potential of eliminating bacterial genes typically used as transformation "markers" which are a cause of concern for some consumers.

Examination of the PPV susceptibility will be undertaken through inoculation of young propagated trees of 'French Prune' in an approved BL3 biocontainment greenhouse facility at USDA-ARS, Ft. Detrick, Maryland. Under these conditions vegetative tissue symptoms can be evaluated and these will be compared to known symptoms on European varieties.
Budwood will be sent to a European collaborator for field tests and observations of fruit symptoms. These fruit symptom observations can be reported to the CDFB in the future but will be beyond the scope of the proposed project timetable.

RESULTS

Project funds were received by ARS and officially made available to R. Scorza on August 15, 2007. A report of project work for the period August 15 – Nov. 1 2007 was submitted in December 2007. The results reported in the current narrative represent results from November 1, 2007 to November 1, 2008.

'French Prune' budwood was kindly provided by Dave Wilson Nursery and by the National Clonal Repository in Davis, CA. *P. domestica* rootstock was bud-grafted and 'French Prune' buds were allowed to produce shoots. As these shoots developed they were harvested for in vitro tissue culture (Fig. 1). During the initial stages of the project we were able to successfully establish shoots of 'French Prune' in vitro which is the first step in developing a clonal regeneration system (Fig. 1).

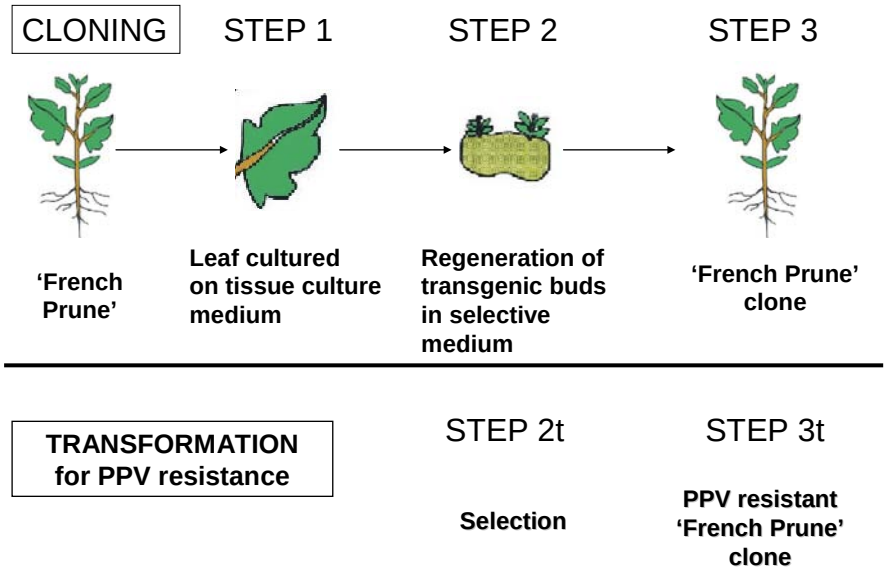

Figure 1. Scheme for the regeneration and transformation of 'French Prune'

A series of factors known to affect regeneration (cloning) were evaluated and optimized for 'French Prune'. These included growth regulators (plant hormones), growth media nutrients, chemicals used to set up the gelling of the tissue culture medium and the ratio of hours darkness to light. Optimization of these factors has led to an average over multiple experiments of 64% of the cultured leaves producing one to several shoots each. This represents a 78% increase over the regeneration that we initially obtained from 'French Prune'. These regenerated shoots were then exposed to a number of treatments to induce further shoot development and rooting. This work led to the production of rooted plants in vitro which were successfully transferred to the greenhouse (Fig. 2). This work demonstrates the success in cloning 'French Prune' from leaves (cloning steps 1 - 3 in Figure 1) which has not been previously reported for this cultivar.

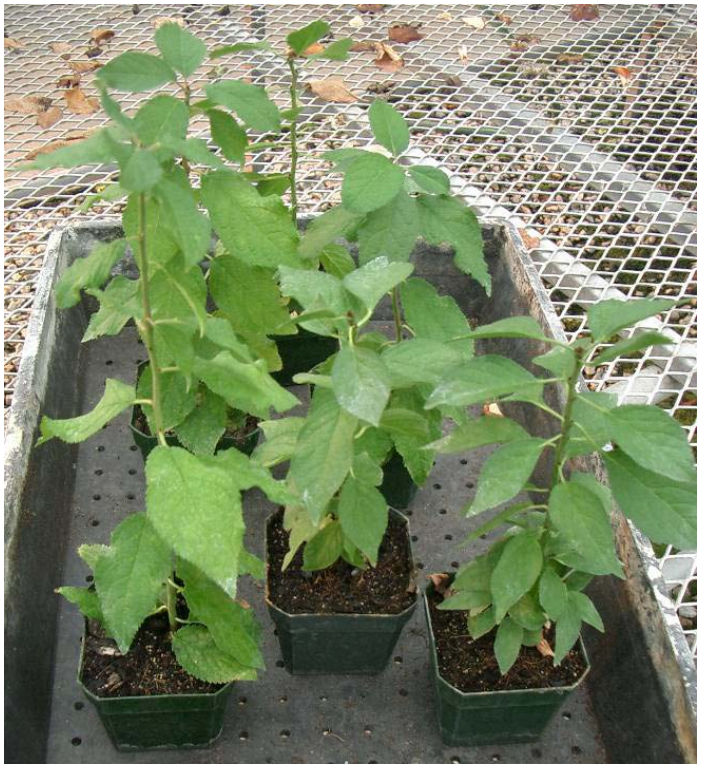

Figure 2. 'French Prune' plants growing in the greenhouse developed from in vitro cloning of leaves.

In addition to the work on regeneration we initiated transformation studies for 'French Prune'. In order to discriminate those regenerated shoots that carry the transferred gene for PPV resistance it was necessary to use a gene specifically for selection so that only the shoots that are transformed will live on the culture medium with the selection agent. We tested a herbicide resistance gene for resistance to Basta (glufosinate) and a gene for resistance to the antibiotic kanamycin, both resistance genes have been approved for use in genetically engineered crops. We found that both were useful in plum for selecting leaf tissue in vitro that was transformed. Based on the long history of safe use for kanamycin, its EPA exemption from tolerance in raw agricultural commodities, and our success with its use in plum transformation from seed material we chose to concentrate on the kanamycin selection system. Additionally, although there is a general wariness of the use of antibiotic resistance markers in the EU, kanamycin resistance has been favorably reviewed for use by the European Food Safety Authority (EFSA) in two separate reports. [In the future we plan to test the possibility of eliminating the antibiotic resistance gene and produce transgenic 'French Prune' plants without selection. We can now do that using plum seed material which regenerates at much higher levels than do 'French Prune' leaves at this point in time.]

Using the kanamycin resistance selection system we have been able to achieve a transformation rate of 90% for in vitro cultured leaves of 'French Prune'. That is, 90% of the leaves that we exposed to transformation treatments produced areas of growth on the selection medium indicating that cells had taken up the genes and could then grow under antibiotic selection. Shoots were developed from these transformed leaves but these shoots subsequently died on the

kanamycin-containing medium indicating that either, they were not originally transformed but were able to survive for a short time on kanamycin or they were chimeras, that is, they were a mix of transformed and non-transformed cells and eventually succumbed to kanamycin. This work demonstrates the accomplishment of transformation step 2t (Fig. 1) but we have not yet achieved step 3t) and this will be a major goal of the project for the 2008-2009 cycle.

In order to test the susceptibility of 'French Prune' to PPV and to evaluate leaf symptoms we inoculated plants with PPV Pennsylvania D strain under containment conditions at the USDA facility at Ft. Detrick Maryland. Molecular analyses (PCR) proved these plants to be infected with PPV (Fig. 3) but they did not produce clear PPV symptoms on leaves. These plants had been aphid inoculated with the D strain isolate from Pennsylvania. While this strain (PENN-3) produces severe symptoms on peach we find that in general it does not produce clear symptoms on plum. We are identifying additional U.S. strains with which to inoculate 'French Prune' based on symptom development on plum.

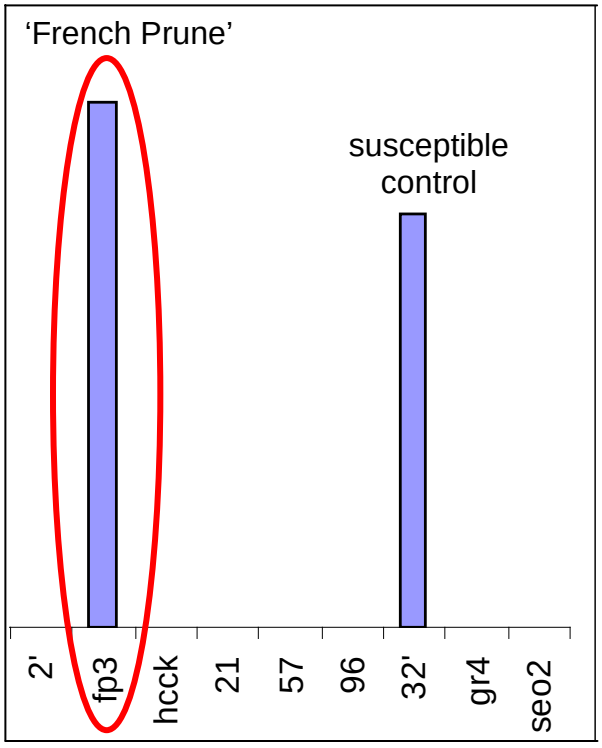

Figure 3. Quantative PCR analysis of 'French Prune' PPV infection. Bars indicate a high level of infection in 'French Prune' and the susceptible control. Absence of bars indicates little of no infection of the resistant test plants.

During the winter of 2007-2008 we had a commitment from a long-time collaborator in Romania to test 'French Prune' for leaf and fruit symptom expression in a test plot in Romania. This plot contains the D, M, and REC (D+M recombinant) strains of PPV and would provide an excellent test of 'French Prune' reaction to PPV infection on leaves and fruit. Although we had a signed and officially stamped permit from Romania to export the material from NRSP-5 to the researcher's institute in Romania, APHIS would not accept the authorization and blocked the

shipment of material. Finally, after it was too late in the year to send budwood, APHIS approved the shipping of the material. We are currently working on a winter 2008-2009 shipment to Romania and have every expectation that this transfer will be successful.