

Deregulation of Plum Pox Resistant Transgenic Plum 'HoneySweet'

Ralph Scorza¹, Jean-Michel Hily¹, Ann Callahan¹, Tadeusz Malinowski²,
Mariano Cambra³, Nieves Capote³, Ioan Zagrai⁴, Vern Damsteegt⁵, Pascal Briard⁶
and Michel Ravelonandro⁶

¹USDA-ARS Appalachian Fruit Research Station, Kearneysville, West Virginia, USA

²Instytut Sadownictwa i Kwiaciarnictwa, Skierniewice, Poland

³Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain

⁴Fruit Research Station, Bistrita, Romania

⁵USDA-ARS Foreign Disease-Weed Science Research Unit, Ft. Detrick, Maryland, USA

⁶Institut de Biologie Vegetale et Moleculaire, INRA, Bordeaux, France

Abstract

Genetic engineering (GE) has the potential to revolutionize fruit tree breeding. It is an approach that can specifically target genetic improvements and allow for the development of novel, useful traits. While GE does not provide a panacea for all of the difficulties associated with fruit tree breeding, it can be a useful approach to increase the efficiency and effectiveness of breeding programs. In spite of the potential utility of GE for fruit tree improvement, the technology has not, to date, been widely exploited in these species. Of over 11,000 field tests of transgenic plants in the United States between 1987 and 2004, less than 1% have involved fruit tree species. Transgenic plum trees that are highly resistant to *Plum pox virus* (PPV) are one example of GE that can be of significant benefit to growers and consumers while providing unique genetic material for use in conventional breeding programs. The development and testing of this plum has spanned 15 years and included researchers in five countries. Currently this plum, 'HoneySweet', is being evaluated for deregulation by U.S. regulatory agencies so that it may possibly be released in the future to breeders and growers who are concerned about the threat of PPV to U.S. stone fruit production.

INTRODUCTION

Plum pox virus (PPV) causes Sharka disease, the most serious virus disease of stone fruits which include peach, nectarine, plum, apricot and cherries. PPV is spread from tree to tree by aphids and through infected budwood. Symptoms of plum pox infection include leaf and fruit yellowing, fruit deformation, premature fruit drop, and when in the presence of other *Prunus* viruses, PPV can cause tree decline. Originally reported from Bulgaria (Atanassov, 1932), plum pox virus has spread throughout Europe where it has caused dramatic production losses (Kölber et al., 2001; Németh, 1994; Roy and Smith, 1994). In the past decade, it has spread from the European continent (see Kölber, 2001) to India, Egypt (Abdel-Ghaffar et al., 1998), Lebanon, Jordan (Al Rwahnih et al., 2001), the Azores, Chile (Herrera et al., 1998), and most recently, the states of Pennsylvania (Levy et al., 2000), New York (<http://www.cals.cornell.edu/cals/public/comm/news/archive/plum-fruit-virus.cfm>) and Michigan (<http://www.aphis.usda.gov/newsroom/content/2006/08/ppvmich.shtml>) in the U.S., Canada (Thompson et al., 2001), Argentina (http://archives.foodsafetynetwork.ca/agnet/2005/5-2005/agnet_may_28.htm) and China (Navratil et al., 2005).

PPV was found in commercial peaches in Pennsylvania in the fall of 1999. Thus far, the eradication program in Pennsylvania alone has cost approximately \$40 million, and 1600 acres of stone fruits have been destroyed. The extent of the infections in New York and Michigan are yet unknown. The potential for PPV to enter California, the major stone fruit producing region in the U.S., is a serious threat to the U.S. stone fruit industry.

PPV is not limited to the commercial *Prunus* species but has been shown to infect most of the native North American *Prunus* species tested including *P. americana*, *besseyi*, *nigra pensylvanica*, *serotina*, *spinosa*, and *virginiana* (Damsteegt et al., 2006). The potential effect of PPV on North American native species is unknown but could present a serious disease problem should it spread from infected orchards to forests.

There are few sources of natural resistance to PPV. A multigenic hypersensitive reaction has been reported in *P. domestica* and a resistant hypersensitive cultivar 'Jojo' has been released (Hartmann and Petruschke, 2002). Several apricot cultivars have been reported to be resistant. Multiple groups are investigating this resistance in apricot and developing molecular markers for use in breeding programs to help facilitate the transfer of resistance to new genotypes (Abernathy et al., 2004; Soriano et al., 2004). It is difficult and long-term to incorporate such resistance into new stone fruit varieties through conventional breeding even when utilizing molecular markers. While the utilization of natural sources of resistance is important for the development of new varieties there is a critical need for complementary approaches to traditional breeding.

RESEARCH TIME-LINE

In 1989 our laboratory (USDA-Kearneysville) began work on the development of resistance to PPV through genetic engineering. Our first studies utilized the papaya ringspot virus (PRV) coat protein (CP) gene (kindly provided by Dr. Dennis Gonsalves) which has been used to develop PRV resistant papayas (Gonsalves, 1998). This virus CP gene had significant homology to the PPV-CP gene. Virus resistance was expected to be CP-mediated (Beachy et al., 1990). The heterologous protection against PPV in plum based on PRV-CP expression was effective for several years in greenhouse tests but after 32 months resistance "broke-down" (Scorza et al., 1995). The PPV-CP gene was isolated, sequenced, and cloned (Ravelonandro et al., 1992) and used for *Agrobacterium*-mediated transformation of plum following the methods of Mante et al. (1991). Again, it was hypothesized that protection would be CP-based. The first two years of the project were dedicated to transferring the gene into plum, producing the genetically engineered (GE) plants, and propagating them for testing. During the following two years greenhouse tests for resistance were conducted at the USDA-ARS BSL3-P containment greenhouse at Ft. Detrick, Maryland. Surprisingly, one transgenic plum plant that appeared highly resistant in greenhouse tests did not express PPV-CP (Ravelonandro et al., 1997; Scorza et al., 2001). This clone "C5", patented as 'HoneySweet', became the focus of research on the mechanism and stability of resistance to PPV. While the C5 clone appeared to be highly resistant in greenhouse tests, field testing was necessary in order to evaluate resistance under typical orchard conditions and in different plum-growing environments. Collaborations were developed with research partners in Europe (Poland, Romania, and Spain) to test this resistant clone in areas where PPV was established. Appropriate field test permits were granted in each country, and field trials were initiated in 1996-1997 which was six to seven years following the initial plum transformations. In the U.S., at USDA-ARS, Kearneysville a field trial was planted under a USDA-Animal and Plant Health Inspection Service (APHIS) permit. This was not to test for resistance since PPV was not present in the U.S. and we could not inoculate plants in the field, but rather to evaluate the trees for their horticultural traits including growth habit and fruit quality, and to initiate risk assessment studies. 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 further confirmed these findings (Malinowski et al., 2006). Investigations of the mechanism of resistance in C5 showed that resistance was through post-transcriptional gene silencing (PTGS) (Scorza et al., 2001; Hily et al., 2004, 2005).

To bring 'HoneySweet' plum to the point where we could consider deregulation based on data field test data and research into the resistance mechanism took approximately 12 years from the time that the first transformations with the PPV-CP gene were initiated. Pre-submission consultations with U.S. regulatory agencies [USDA Animal and Plant Health Inspection Service (APHIS), the U.S. Food and Drug Administration (FDA) and the U.S. Environmental Protection Agency (EPA)] began in 2003. APHIS has general jurisdiction over the planting of GE plants. FDA has jurisdiction over GE plants used as food, and EPA regulates GE crop plantings of over ten acres and regulates GE plants that produce molecules that protect plants against pests (protection against PPV in the case of

There are few sources of natural resistance to PPV. A multigenic hypersensitive reaction has been reported in *P. domestica* and a resistant hypersensitive cultivar 'Jojo' has been released (Hartmann and Petruschke, 2002). Several apricot cultivars have been reported to be resistant. Multiple groups are investigating this resistance in apricot and developing molecular markers for use in breeding programs to help facilitate the transfer of resistance to new genotypes (Abernathy et al., 2004; Soriano et al., 2004). It is difficult and long-term to incorporate such resistance into new stone fruit varieties through conventional breeding even when utilizing molecular markers. While the utilization of natural sources of resistance is important for the development of new varieties there is a critical need for complementary approaches to traditional breeding.

RESEARCH TIME-LINE

In 1989 our laboratory (USDA-Kearneysville) began work on the development of resistance to PPV through genetic engineering. Our first studies utilized the papaya ringspot virus (PRV) coat protein (CP) gene (kindly provided by Dr. Dennis Gonsalves) which has been used to develop PRV resistant papayas (Gonsalves, 1998). This virus CP gene had significant homology to the PPV-CP gene. Virus resistance was expected to be CP-mediated (Beachy et al., 1990). The heterologous protection against PPV in plum based on PRV-CP expression was effective for several years in greenhouse tests but after 32 months resistance "broke-down" (Scorza et al., 1995). The PPV-CP gene was isolated, sequenced, and cloned (Ravelonandro et al., 1992) and used for *Agrobacterium*-mediated transformation of plum following the methods of Mante et al. (1991). Again, it was hypothesized that protection would be CP-based. The first two years of the project were dedicated to transferring the gene into plum, producing the genetically engineered (GE) plants, and propagating them for testing. During the following two years greenhouse tests for resistance were conducted at the USDA-ARS BSL3-P containment greenhouse at Ft. Detrick, Maryland. Surprisingly, one transgenic plum plant that appeared highly resistant in greenhouse tests did not express PPV-CP (Ravelonandro et al., 1997; Scorza et al., 2001). This clone "C5", patented as 'HoneySweet', became the focus of research on the mechanism and stability of resistance to PPV. While the C5 clone appeared to be highly resistant in greenhouse tests, field testing was necessary in order to evaluate resistance under typical orchard conditions and in different plum-growing environments. Collaborations were developed with research partners in Europe (Poland, Romania, and Spain) to test this resistant clone in areas where PPV was established. Appropriate field test permits were granted in each country, and field trials were initiated in 1996-1997 which was six to seven years following the initial plum transformations. In the U.S., at USDA-ARS, Kearneysville a field trial was planted under a USDA-Animal and Plant Health Inspection Service (APHIS) permit. This was not to test for resistance since PPV was not present in the U.S. and we could not inoculate plants in the field, but rather to evaluate the trees for their horticultural traits including growth habit and fruit quality, and to initiate risk assessment studies. 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 further confirmed these findings (Malinowski et al., 2006). Investigations of the mechanism of resistance in C5 showed that resistance was through post-transcriptional gene silencing (PTGS) (Scorza et al., 2001; Hily et al., 2004, 2005).

To bring 'HoneySweet' plum to the point where we could consider deregulation based on data field test data and research into the resistance mechanism took approximately 12 years from the time that the first transformations with the PPV-CP gene were initiated. Pre-submission consultations with U.S. regulatory agencies [USDA Animal and Plant Health Inspection Service (APHIS), the U.S. Food and Drug Administration (FDA) and the U.S. Environmental Protection Agency (EPA)] began in 2003. APHIS has general jurisdiction over the planting of GE plants. FDA has jurisdiction over GE plants used as food, and EPA regulates GE crop plantings of over ten acres and regulates GE plants that produce molecules that protect plants against pests (protection against PPV in the case of

'HoneySweet'). An application for determination of non-regulatory status was submitted to APHIS in April 2006 and it was accepted for review. In May, 2006, the petition was posted on the internet for 60 days of public comment. A determination by APHIS is pending. Data packages will also be submitted to FDA and EPA in 2006. These submissions are taking place 16 years following the initial transformations.

CONCLUSIONS

'HoneySweet' has proven to be highly resistant to PPV for over seven years in the three European field tests under heavy infection pressure. No tree has ever been infected by aphids, and trees inoculated by grafting with PPV-infected budwood have shown only very mild infection near the graft union with few symptoms that disappear as the growing season progresses (Hily et al., 2004; Malinowski et al., 2006). We found that the resistance can be transferred to seedlings through cross-hybridization (breeding) (Ravelonandro et al., 1998; Scorza et al., 1998).

'HoneySweet' fruit quality is excellent, and productivity appears to be very good. Fruit weigh an average of 2.1 oz and measure and average of 1.73 x 2.0 inches. They are freestone with a small area of clinginess. Sugar content as measured by °brix is 21-22°. The tree has an upright growth habit and is spur type. 'HoneySweet' is self-incompatible.

If 'HoneySweet' is deregulated by APHIS, FDA, and EPA, this will mean that 'HoneySweet' could be grown and marketed in the U.S. as any other plum variety without special permits. More likely, if it is deregulated, it will be used for research and breeding to develop other PPV resistant plum varieties. PPV is currently under control and being eradicated in the U.S., so currently there is no grower demand for this plum. However, heightened demand could result from further detections of PPV in the U.S., particularly in California where most of the U.S. dried plum industry is located. This would be a very serious problem should it occur and resistant varieties would be needed, not only 'HoneySweet', but also others with specific fruit qualities, adaptation, and ripening dates. In anticipation of the potential spread of PPV to other regions of the U.S. breeding with 'HoneySweet' would be beneficial and relatively rapid since the presence of the resistance gene in 'HoneySweet' seedlings is easily assayed.

The resistance technology (PTGS) tested in 'HoneySweet' can be used to develop other resistant stone fruits, such as peach, apricot, Japanese plum, and cherry, which are all susceptible to a greater or lesser extent to PPV. In general, apricots and plums are particularly susceptible. Since our work with 'HoneySweet' has demonstrated the effectiveness of GE technology in producing high levels of virus resistance, the time necessary to produce the next generation of resistant plants should be shorter, but it will still require years of work. As more information on sources and mechanisms of natural resistance are obtained, transgenic resistance can be combined with naturally occurring resistance to produce trees with a broader type of resistance that is based on several genetic mechanisms. This applied research is needed to form a germplasm base to begin to protect stone fruit production from PPV. Direct transformation of stone fruit varieties in place of seed-based transformation is also needed for the most efficient application of genetically engineered resistance.

'HoneySweet' is the culmination of years of research and development, but it is only the beginning of an effort to produce PPV resistant stone fruit varieties that will secure the welfare of fruit growers and the supply of safe and affordable fruit.

Literature Cited

- Abdel-Ghaffar, M.H., Abo El-Nasr, M.A. and Hari, V. 1998. Studies on an apricot strain of plum pox potyvirus isolated from El Amar, Egypt. *Acta Hort.* 472:385-391.
- Abernathy, D., Zhebentyayeva, T., Vilanova, S., Badenes, M.L., Salava, J., Polák, J., Krška, B. and Damsteegt, V.D. 2004. Molecular genetic mapping of the Plum pox virus resistance genes in apricot. *Acta Hort.* 657:283-288.
- Al Rwahnih, M., Myrta, A., Di Terlizzi, B. and Boscia, D. 2001. First record of plum pox virus in Jordan. *Acta Hort.* 550:141-144.

- Atanassov, D. 1932. Plum pox. A new virus disease. *Ann. Univ. Sofia, Fac. Agric. Silv.* 11:49-69.
- Beachy, R.N., Loesh-Fries, S. and Tumer, N.E. 1990. Coat protein-mediated resistance against virus infection. *Ann. Rev. Phytopath.* 28:451-474.
- Damsteegt, V.D., Scorza, R., Stone, A.L., Schneider, W.L., Webb, K., Demuth, M. and Gildow, F.E. 2006. *Prunus* host range of *Plum pox virus* (PPV) in the United States by aphid and graft inoculation. *Plant Dis.* 90 (Accepted Aug 12, 2006).
- Gonsalves, D. 1998. Control of papaya ringspot virus in papaya – A case study. *Ann. Rev. Phytopath.* 36:415-437.
- Hartmann, W. and Petruschke, M. 2002. Sharka resistant plums and prunes by utilization of hypersensitivity. *Acta Hort.* 538:391-395.
- Herrera, G., Sepulveda, P. and Madariaga, M. 1998. Survey of sharka disease (*Plum pox virus*) on stone fruit trees in Chile. *Acta Hort.* 472:393-399.
- Hily, J.-M., Scorza, R., Malinowski, T., Zawadzka, B. and Ravelonandro, M. 2004. Stability of gene silencing-based resistance to *Plum pox virus* in transgenic plum (*Prunus domestica* L.) under field conditions. *Transgenic Res.* 13:427-436.
- Hily, J.-M., Scorza, R., Webb, K. and Ravelonandro, M. 2005. Accumulation of the long class of siRNA is associated with resistance to *Plum pox virus* in a transgenic woody perennial plum tree. *MPMI* 18:794-799.
- Kölber, M. 2001. Workshop on Plum pox. *Acta Hort.* 550:153-157.
- Kölber, M., Nemeth, M., Dulic-Markovic, I., Isac, M., Malinowski, T., Zawadzka, B., Myrta, A., Prichodko, Y., Topchiiska, M., Chernets, A., Kalashian, Y., Glasa, M., Kriska, B., Minoiu, N., Navratil, M. and Slovakova, L. 2001. Current situation of Plum pox disease on stone fruit species in Middle and Eastern Europe. *Acta Hort.* 550:73-78.
- Levy, L., Damsteegt, V. and Welliver, R. 2000. First Report of *Plum Pox Virus* (Sharka disease) in *Prunus persica* in the United States. *Plant Dis.* 8:202.
- Malinowski, T., Cambra, M., Capote, N., Zawadzka, B., Gorris, M.T., Scorza, R. and Ravelonandro, M. 2006. Field trials of plum clones transformed with the *Plum pox virus* coat protein (PPV-CP) gene. *Plant Dis.* 90:1012-1018.
- Mante, S., Morgens, P.H., Scorza, R., Cordts, J.M. and Callahan, A.M. 1991. *Agrobacterium*-mediated transformation of plum (*Prunus domestica* L.) hypocotyls slices and regeneration of transgenic plants. *BioTechnology* 9:853-857.
- Navratil, M. and Safarova, D. 2005. First incidence of *Plum Pox Virus* on apricot trees in China. *Plant Dis.* 89:338.
- Németh, M. 1994. History and importance of plum pox in stone-fruit production. *Bulletin OEPP-EPPO* Bulletin 24:525-536.
- Ravelonandro, M., Monsion, M., Tycheney, P.Y., Delbos, R. and Dunez, J. 1992. Construction of a chimeric viral gene expressing *plum pox virus* coat protein. *Gene* 120:167-173.
- Ravelonandro, M., Scorza, R., Bachelier, J.C., Labonne, G., Levy, L., Damsteegt, V., Callahan, A.M. and Dunez, J. 1997. Resistance of transgenic *Prunus domestica* to plum pox virus infection. *Plant Dis.* 81:1231-1235.
- Ravelonandro, M., Scorza, R., Renaud, R. and Salesses, G. 1998. Transgenic plums resistant to plum pox virus infection and preliminary results of cross-hybridization. *Acta Hort.* 478:67-71.
- Roy, A.S. and Smith, I.M. 1994. Plum pox situation in Europe. *Bulletin OEPP-EPPO* Bulletin 24:515-524.
- Scorza, R., Levy, L., Damsteegt, V., Yepes, L.Z., Cordts, J., Hadidi, A., Slightom, J. and Gonsalves, D. 1995. Transformation of plum with the *Papaya ringspot virus* coat protein gene and reaction of transgenic plants to *Plum pox virus*. *J. Amer. Soc. Hort. Sci.* 120:943-952.
- Scorza, R., Callahan, A., Levy, L., Damsteegt, V. and Ravelonandro, M. 1998. Transferring potyvirus coat protein genes through hybridization of transgenic plants to produce plum pox virus resistant plums (*Prunus domestica* L.). *Acta Hort.* 472:421-425.

- Scorza, R., Callahan, A., Levy, L., Damsteegt, V., Webb, K. and Ravelonandro, M. 2001. Post-transcriptional gene silencing in plum pox virus resistant transgenic European plum containing the plum pox potyvirus coat protein gene. *Transgenic Res.* 10:201-209.
- Soriano, J.M., Vilanova, S., Romero, C., Llacer, G. and Badenes, M.L. 2004. Cloning and characterization of NBS-LRR sequences in apricot. *Acta Hort.* 663:153-156.
- Thompson, D., McCann, M., MacLeod, M., Lye, D., Green, M. and James, D. 2001. First report of plum pox potyvirus in Ontario, Canada. *Plant Dis.* 85:97.