Biotech/GM Crops in Horticulture: Plum cv. HoneySweet Resistant to Plum Pox Virus

Jaroslav Polak, Jiban Kumar, Boris Krška, Michel Ravelonandro

Biotech/GM Crops in Horticulture: Plum cv. HoneySweet Resistant to *Plum Pox Virus*

Jaroslav POLÁK[1], Jiban KUMAR[1], Boris KRŠKA[2] and Michel RAVELONANDRO[3]

[1]Department of Virology, Division of Plant Health, Crop Research Institute, Prague, Czech Republic; [2]Mendel University inBrno, Faculty of Horticulture, Lednice, Czech Republic; [3]INRA-Bordeaux, Villenave d'Ornon, France

Abstract

Polák J., Kumar J., Krška B., Ravelonandro M. (2012): **Biotech/GM crops in horticulture: Plum cv. HoneySweet resistant to *Plum pox virus***. Plant Protect. Sci., **48** (Special Issue): S43–S48.

Commercialisation of Biotech/GM (Biotech) crops started in 1995. Not only field crops, but also horticultural transgenic crops are under development and are beginning to be commercialised. Genetic engineering has the potential to revolutionise fruit tree breeding. The development of transgenic fruit cultivars is in progress. Over the past 20 years an international public sector research team has collaborated in the development of HoneySweet plum which is highly resistant to *Plum pox virus* (PPV) the most devastating disease of plums and other stone fruits. HoneySweet was deregulated in the USA in 2010. HoneySweet (aka C5) has been evaluated for eleven years (2002–2012) in a regulated field trial in the Czech Republic for the resistance to PPV, *Prune dwarf virus* (PDV), and *Apple chlorotic leaf spot virus* (ACLSV), all of them being serious diseases of plum. Even under the high and permanent infection pressure produced through grafting, PPV has only been detected in HoneySweet trees in several leaves and fruits situated close to the point of inoculum grafting. The lack of infection spread in HoneySweet demonstrates its high level of PPV resistance. Co-infections of PPV with PDV and/or ACLSV had practically no influence on the quantity and quality of HoneySweet fruit which are large, sweet, and of a high eating quality. In many respects, they are superior to the fruits of the well-known cultivar Stanley. Many fruit growers and fruit tree nurseries in the Czech Republic are supportive of the deregulation of HoneySweet plum to help improve the plum production and control the spread of PPV.

Keywords: genetic modifications; fruit trees; GM plum; Sharka disease; resistance

The first Biotech/GM (Biotech) crops, cotton (Monsanto) and potato (Syngenta) were commercialised in 1995. Clearly, the need for food security and sustainability extends to horticultural crops such as fruits and vegetables which are the main sources of nutrients and healthful compounds necessary for health and well-being of the world population. Biotech zuccini and squash resistant to *Zuccini yellow mosaic virus, Watermelon mosaic virus,* and *Cucumber mosaic virus* are grown in the USA (Dias & Ortiz 2011).The research of the transformation of the potato, cucumber, carrot, egg plant, sweet corn, and other vegetables in many countries of the world is aimed at the resistance to viruses, bacteria, fungi, insects, at the tolerance to herbicides, at the improvement of economic properties, prolongation of the consumption time, improvement of nutrition values, seedlessness of fruits. The development of transgenic fruit cultivars is in progress. Papaya resistant to *Papaya mosaic virus* is grown in USA and China (James 2011). Biotech grapevine resistant to viral, bacterial, and fungal diseases with abiotic stress tolerance and health benefits was developed in South Africa.

Supported by the Ministry of Agriculture of the Czech Republic, Projects No. QI101A123 and No. 0002700604.

Biotech banana, apple, pear, and strawberry cultivars are under the development.

The result of the international research done over the past 20 years is the development of HoneySweet plum highly resistant to PPV. GM plum HoneySweet resistant to *Plum pox virus* (PPV) was deregulated in USA in 2010. Plums (*Prunus domestica*) are an important source of vitamins, minerals, and phytonutrients and contain specific compounds that support good digestive function and bone health. Sharka disease is the most devastating disease of plum and is responsible for the reduction or loss of the plum production in many areas of Europe (Cambra *et al*. 2006). In the past 22 years of research by an international team of public sector scientists, a GM plum highly resistant to PPV was developed and thoroughly tested in the greenhouse and field, in the USA and Europe – the Czech Republic, France, Poland, Romania, and Spain for the resistance to PPV and for environmental safety (Scorza *et al*. 1994; Ravelonandro *et al*. 1997, 2000, 2002; Hily *et al*. 2004, 2007; Polák *et al*. 2005, 2008a,b; Malinowski *et al*. 2006; Capote *et al*. 2007, 2008; Zagrai *et al*. 2008a,b, 2010). An original trial of a high and permanent infection pressure of PPV-Rec alone and in combinations with *Prune dwarf virus* (PDV) and *Apple chlorotic leafspot virus* (ACLSV) was initiated in the Czech Republic (Polák *et al*. 2008a,b). The transgenic plum trees were evaluated during the years 2002–2012. Here, we present a summary of the results of eleven year testing of HoneySweet plum (clone C5) and the results of three year testing of the fruits quality (2010–2012) under the high and permanent infection pressure coming both from the graft inoculation and natural aphid vectors, and discuss the implications of the work with HoneySweet in terms of its potential for utilisation in the EU.

MATERIAL AND METHODS

Field trial, transgenic plum trees, virus inoculations. Original plum clone C5 buds from USA (USDA-ARS, Kearneysville) were grafted onto the virus-free rootstocks of St. Julien in 2002, and 55 grafted *P. domestica* clone C5/St. Julien trees were obtained. Each inoculation treatment PPV-Rec, PPV-Rec + ACLSV, PPV-Rec + PDV, PPV-Rec + ACLSV + PDV, consisted of 11 C5 trees. The inoculated non GM controls and non-inoculated control and C5 trees were included and a plantation was established (Figure 1). PPV-Rec, ACLSV, and PDV infected buds were allowed to grow throughout the eleven years period of evaluation. The transgenic clone C5 part of each tree remained eleven years under a very strong graft inoculation pressure.

Figure 1. Plantation of HoneySweet trees in Czech Republic (Orig. J. Polák)

Evaluation of leaf and fruit symptoms, quality of fruits. All trees were evaluated every year in the period from May to September (2002–2012) for the presence of viral symptoms in leaves. Fruit symptoms were evaluated in July and August 2010–2012 (the first few fruits were produced in 2009) a short time before ripening when fruits were still firm and at full ripening. In 2010–2012 were included the overall fruit uniformity, attractiveness, weight, length, diameter, flesh thickeness, fruit shape, skin colour, flesh colour, flesh firmness, flavour, freeness of flesh from the stone, total soluble solids, total titratable acidity, stone size, weight and stone/flesh ratio, and dry weight of fruits harvested from the trees of clone C5 inoculated with PPV-Rec, PPV-Rec + ACLSV, PPV-Rec + PDV, PPV-Rec + ACLSV + PDV, and from the non-inoculated control trees of clone C5, Stanley, and Domácí švestka.

Serological detection of viruses. ELISA testing of the leaves was performed every year in June. Fruits were evaluated in August 2010–2012. Polyclonal antibodies raised against PPV, ACLSV, and PDV (Bioreba, Reinach, Switzerland) were used in DAS-ELISA (Clark & Adams 1977). The leaf samples were extracted in phosphate-buffered saline. The relative concentration of PPV-Rec was determined by semiquantitative DAS-ELISA in the samples prepared from the symptomatic leaves in June 2005 and 2007. The relative concentration of PPV protein was established by determining the

lowest dilution of leaf or fruit samples with the positive reaction in semiquantitative DAS-ELISA (Albrechtová *et al*. 1986).

Detection of viruses by reverse transcription-polymerase chain reaction (RT-PCR). 100 mg of ground leaf or fruit tissue were used for total RNA extraction by using RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the procedure recommended by the manufacturer. PPV-Rec was detected by RT-PCR using the primer pair mD5/mM3 as described by Šubr *et al*. (2004). For PDV and ACLSV, the primers were used as described by Jarošová and Kundu (2010).

RESULTS AND DISCUSSION

No PPV symptoms appeared in the leaves of the transgenic plum clone C5 HoneySweet trees in the first year after the graft-inoculation with PPV-Rec. PPV symptoms appeared only in the leaves that emerged from the infected buds (IB). Mild diffuse spots and rings appeared two years after the inoculation in some basal leaves of HoneySweet trees inoculated with PPV-Rec, and in those inoculated with the virus combinations PPV-Rec + ACLSV, PPV-Rec + PDV, and PPV-Rec + ACLSV + PDV (Polák *et al*. 2005). PPV presence in these basal leaves was confirmed by ELISA and RT-PCR. A reduction of symptoms was observed beginning in the third year after the virus inoculation. PPV symptoms were observed only in several basal leaves and the symptoms were milder in each following year (Polák *et al*. 2008a).

Further reduction of PPV symptoms was observed in years 2009–2012, and during the vegetation period from June to September. No PPV symptoms were found in leaves in 2012, and the presence of PPV was not proved by ELISA. No differences in the intensity of PPV leaf symptoms between different virus combinations were observed in the years 2004–2012. No symptoms of PDV and ACLSV appeared during the vegetative periods of 2002–2012. PDV was not detected by ELISA in transgenic parts of trees inoculated with PPV-Rec + PDV and PPV-Rec + PDV + ACLSV. The presence of PDV was dubious by RT-PCR. PDV was detected by ELISA and RT-PCR only in leaves growing from the IB. ACLSV was detected by ELISA and RT-PCR in leaves of transgenic parts of the trees inoculated with PPV-Rec + ACLSV and PPV-Rec + PDV + ACLSV. No symptoms of PPV, PDV, and ACLSV appeared in the leaves of the non-inoculated control trees of HoneySweet throughout the experiment, and PPV, PDV, and ACLSV were not detected by DAS-ELISA and RT-PCR. The growth of the non-inoculated HoneySweet control trees was more vigorous in comparison with those inoculated with PPV and the combinations with PDV and ACLSV. This may have been due in the whole or part to the competition by the extensive growth of IB shoots growing from the inoculated HoneySweet trees (Figure 2). The severe PPV symptoms which appeared first in 2003 in IB leaves growing from the buds infected with PPV-Rec appeared again every year (2004–2012) with the same intensity.

The relative concentration of PPV-Rec in the symptomatic leaves of HoneySweet determined by semiquantitative DAS-ELISA fluctuated from 1.56×10^{-2} to 9.76×10^{-4} in 2005 and from 5.0×10^{-1} to 7.81×10^{-3} in 2007. There were no significant differences in the relative concentration of PPV between the combinations of the inoculated viruses. The relative concentration of PPV in the leaves of IB shoots was at least thirty times higher as compared to the symptomatic leaves of HoneySweet.

Pomological evaluation of the external and internal characteristics of the fruits (Figure 3) harvested from non-graft-inoculated HoneySweet trees, Stanley, and Domácí švestka trees, and from HoneySweet trees growing eleven years under the high and permanent infection pressure by PPV-Rec, PPV-Rec + PDV, PPV-Rec + ACLSV, PPV-Rec + ACLSV + PDV demonstrated the high quality of HoneySweet fruits. The PPV presence was proved by ELISA in several fruits situated close to the place of IB grafting in 2010 and 2011 only. All the fruits were PPV free as found by ELISA in 2012. Three-year results indicate that the characteristics of HoneySweet fruits harvested from the control virus non-inoculated trees are well within the range of the characteristics of control cultivars Stanley and Domácí švestka and are of higher quality in some characteristics. The fruits harvested from HoneySweet trees inoculated with PPV-Rec + ACLSV + PDV, PPV-Rec + PDV, PPV-Rec + ACLSV, and PPV-Rec were comparable with the fruits from the control healthy HoneySweet trees indicating that there was little, if any, effect of the virus inoculations on the fruit quality of HoneySweet.

HoneySweet plum trees resistant to PPV remained virus-free under the natural aphid-vectored infection pressure throughout this eleven-year study. This is in agreement with the results obtained

Figure 2. HoneySweet tree with (a) non-transgenic and (b) cutted away non-transgenic PPV infected bottom part (Orig. J. Polák)

in France and Romania (Ravelonandro *et al.* 1997, 2002), and in Spain and Poland (Malinowski *et al.* 2006). Original results were obtained when graft inoculated trees were exposed to a very high infection pressure with IB being allowed to reach the size of 20–30% of the supporting HoneySweet tree (Figure 2). Under this high and permanent virus pressure, HoneySweet trees showed PPV symptoms and positive serological and molecular tests on some basal leaves only, and even these symptoms subsided during the growing season. Most trees were without any leaf symptoms in 2010 and 2011, and no symptoms appeared in leaves in 2012. ACLSV infection did not appear to affect PPV symptoms and PDV infection could not be detected in HoneySweet throughout the course of the study despite the graft inoculation. The evaluations of the fruit quality of the graft inoculated and non-inoculated HoneySweet trees, maintained for eleven years under the high and permanent infection pressure by PPV, ACLSV, and PDV, confirmed not only the high resistance of HoneySweet to PPV, but also suggested that HoneySweet fruits maintain their quality and healthful properties when exposed not only to PPV but also to ACLSV and PDV.

Figure 3. Fruits of plum HoneySweet (Orig. J. Polák)

The regulatory process in the USA for HoneySweet was successfully completed in 2010. The strong international cooperation between public sector scientists in Europe and the USA and the approval of HoneySweet in the USA warrant the submission of HoneySweet for regulatory consideration in the EU. The ability to grow HoneySweet

plum in the Czech Republic would contribute to the viability of the plum production by Czech growers and support the producers of the products that depend upon a supply of plums including producers of plum brandy. The cultivation of HoneySweet in the Czech Republic and other European countries would represent a unique opportunity to establish PPV free orchards and to grow high quality fruits for the benefit of growers and consumers.

Acknowledgements. Authors are in debt to Mrs M. Ducháčová and J. Pívalová for technical assistance.

References

Albrechtová L., Karešová R., Pluhař Z., Balcarová E. (1986): ELISA method used for the evaluation of resistance of plum cultivars to plum pox virus. In: Proceedings 10th Conference Plant Protection, Brno, Czech Republic: 203–204.

Cambra M., Capote N., Myrta A., Llacer G. (2006): *Plum pox virus* and the estimated costs associated with sharka disease. OEPP/EPPO Bulletin, **36**: 202–204.

Capote N., Monzo C., Urbaneja A., Perez-Panades J., Carbonell E., Ravelonandro M., Scorza R., Cambra M. (2007): Risk assessment of the field release of transgenic European plums susceptible and resistant to *Plum pox virus*. ITEA, **103**: 156–167.

Capote N., Perez-Panades J., Monzo C., Carbonell E., Urbaneja A., Scorza R., Ravelonandro M., Cambra M. (2008): Assessment of the diversity and dynamics of *Plum pox virus* and aphid populations in transgenic European plums under Mediterranean conditions. Transgenic Research, **17**: 367–377.

Clark M.F., Adams A.N. (1977): Characteristic of the microplate method of enzyme-linked immunosorbent assay for the detection of plant virus. Journal of Genetic Virology, **34**: 51–57.

Dias A.S., Ortiz R. (2011): Transgenic vegetables for 21st century horticulture. In: Book of Abstracts: Genetically Modified Organisms in Horticulture Symposium. September 12–16, 2011, Nelspruit, South Africa: **27** (Abstr.)

Hily J. M., Scorza R., Malinowski T., Zawadzka B., Ravelonandro M. (2004): Stability of gene silencing-based resistance to *Plum pox virus* in transgenic plum (*Prunus domestica* L.) under field conditions. Transgenic Research, **13**: 427–436.

Hily J.M., Ravelonandro M., Damsteegt V., Bassett C., Petri C., Liu Z., Scorza R. (2007): *Plum pox virus* coat protein gene intron-hairpin-RNA (ihpRNA) constructs provide resistance to *Plum pox virus* in *Nicotiana benthamiana* and *Prunus domestica*. Journal of American Society of Horticultural Sciences, **132**: 850–858.

James C. (2011): Global Status of Commercialized Biotech/GM Crops: 2011. ISAAA Brief No. 43. The International Service for the Acquisition of Agri-biotech Applications (ISAAA), Ithaca, USA.

Jarošová J., Kundu J.K. (2010): Simultaneous detection of stone fruit tree viruses by one-step multiplex RT-PCR. Scientia Horticulture, **125**: 68–72.

Malinowski, T., Cambra M., Capote N., Zawadzka, B., Gorris M.T., Scorza R., Ravelonandro M. (2006): Field trials of plum clones transformed with the *Plum pox virus* coat protein (PPV-CP) gene. Plant Disease, **90**: 1012–1018.

Polák J., Pívalová J., Jokeš M., Svoboda J., Scorza R., Ravelonandro M. (2005): Preliminary results of interactions of *Plum pox virus* (PPV), *Prune dwarf virus* (PDV), and *Apple chlorotic leafspot virus* (ACLSV) with transgenic plants of plum *Prunus domestica*, clone C5 grown in an open field. Phytopathologia Polonica, **36**: 115–122.

Polák J., Pívalová J., Kumar-Kundu J., Jokeš M., Scorza R., Ravelonandro M. (2008a): Behaviour of transgenic *Plum pox virus*-resistant *Prunus domestica* L. clone C5 grown in the open field under a high and permanent infection pressure of the PPV-Rec strain. Journal of Plant Pathology, **90** (Suppl. 1): S1.33–S1.36.

Polák J., Kumar-Kundu J., Pívalová J., Scorza R., Ravelonandro M. (2008b): Interactions of *Plum pox virus* strain Rec with *Apple chlorotic leaf spot* and *Prune dwarf viruses* in field growing transgenic plum *Prunus domestica* L., clone C. Plant Protection Science, **44**: 1–5

Ravelonandro M., Scorza R., Bachelier J.C., Labbone G., Lery L., Damsteegt V., Callahan A.M., Dunez J. (1997): Resistance of transgenic plums (*Prunus domestica* L.) to *Plum pox virus* infection. Plant Disease, **81**: 1231–1235.

Ravelonandro M., Scorza R., Callahan A., Levy L., Jacquet C., Monsion M., Damsteegt V. (2000): The use of transgenic fruit trees as a resistance strategy for virus epidemics: the plum pox (sharka) model. Virus Research, **71**: 63–69.

Ravelonandro M., Scorza R., Minoiu N., Zagrai I., Platon I. (2002): Field tests of transgenic plums in Romania. Sanatatea Plantelor (Special Edition): 16–18.

Scorza R., Ravelonandro M., Callahan A.M., Cordts J.M., Fuchs M., Dunez J., Gonsalvez D. (1994): Transgenic plums (*Prunus domestica* L.) express the plum pox virus coat protein gene. Plant Cell Reports, **14**: 18–22.

Šubr Z., Pittnerová S., Glasa M. (2004): A simplified RT-PCR-based detection of recombinant *Plum pox virus* isolates. Acta Virologica, **48**: 173–176.

Zagrai I., Ravelonandro M., Gaboreanu I., Ferencz B., Scorza R., Zagrai L., Kelemen B., Pamfil D., Popescu O. (2010): Transgenic plums expressing the *Plum pox virus* (PPV) coat protein gene do not assist the development of PPV recombinants under field conditions. Journal of Plant Pathology, **93**: 159–165.

Zagrai I., Ravelonandro M., Scorza R., Minoiu N., Zagrai L. (2008a): Field release of transgenic plums in Romania. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies, **65**: 358–365.

Zagrai I., Zagrai L., Ravelonandro M., Gaboreanu I., Pamfil D., Ferencz B., Popescu O., Scorza R., Capote N. (2008b): Environmental impact assessment of transgenic plums on the diversity of *Plum pox virus* populations. Acta Horticulturae (ISHS) 781: 309–318.

Received for publication May 23, 2012
Accepted after corrections October 30, 2012

Corresponding author:

Doc. Ing. Jaroslav Polák, DrSc., Výzkumný ústav rostlinné výroby, v.v.i., odbor rostlinolékařství, oddělení virologie, 161 06 Praha 6-Ruzyně, Česká republika
tel. + 420 233 022 315, e-mail: polak @vurv.cz