Oral Session VII

Pathogen-derived technologies for improving Plum Pox Virus resistance of transgenic plum (*Prunus domestica* L.)

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Stone fruit trees are valuable owing to quality of their fruits and as the source of hard wood in all world. Virus and fungus diseases are main dangers for cultivatione of the stone fruit culture. Plum Pox Virus (PPV), causal agent of Sharka disease, is currently being considered as the most infectious pathogen in peaches and nectarine, apricots, plums and cherry. Considering the severity of the disease, complicated control of its spread, and lack of resistant varieties, the necessity of resistant cultivars is apparent.

There are some approaches to improving plant resistance to viral pathogens in modern biotechnology. Pathogen-derived resistance (PDR), based on virus resistance by sense (co-supression) and antisense virus gene expression, or expression of self-complementary fragments of virus gene (RNA-silencing), is widespread at the last time. PDR are divided by two types: transgenic proteinmediated resistance and RNA-mediated resistance. Protein-mediated resistance is occurred due to accumulation of viral protein in plant cell. Post-transcription gene silencing (PTGS), or RNA-silencing, is realized at RNA-mediated virus resistance. RNA-silencing mechanism is cleaving of double-stranded RNAs (dsRNA) result in small interfering RNAs (siRNA), which are template for cleaving homologous molecules of mRNAs.

For improving the plants resistance to plum pox virus (PPV) two technologies were based on co-supression gene and RNA-silencing. Binary vector pCamPPVcp which contained the selective hpt gene and ppv-cp gene in sense-orientation (driven by double 35S promoter). Vector pCamPPVRNAi contained selfcomplementary fragments of gene ppv-cp driven by double 35S promoter and the hpt and gus genes.

Seven independent transgenic lines with ppv-cp gene and five transgenic lines with a inverted repeats of ppv-cp gene fragment were produced in our laboratory. Stable integration of genes into genome of plants was confirmed by PCR analyses. The accumulation of coat protein was evaluated by Western blot assay.

The transgenic shoots were rooted and acclimatized to the greenhouse.

The hairpin structure of the Plum pox virus coat protein gene in HoneySweet C5 plum is responsible for PPV resistance

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The genetically engineered plum ‘HoneySweet’ (aka C5) has proven to be highly resistant to Plum pox virus (PPV) for over 10 years in field trials. The original vector used for transformation to develop ‘HoneySweet’ carried a single sense sequence of the full length ppv-cp gene, yet the resistance mechanism of ‘HoneySweet’ was found to be based on post-transcriptional gene silencing (PTGS). Sequencing of the transgene insert revealed an inverted repeat of the PPV-CP sequence in ‘HoneySweet’. We hypothesized that this structure, acting as a hairpin (hp), was responsible for PTGS of the transgene and of viral CP which resulted in a high level of resistance to PPV infection. In order to test this hypothesis the hpPPV-CP insert was cloned from ‘HoneySweet’ and transferred into ‘Bluebyrd’ plum seedlings through Agrobacterium tumefaciens transformation of hypocotyl slices. Two versions of the hpPPV-CP insert were tested. One spanned the CP inverted repeat only (minimal construct) and the other

spanned the inverted repeat plus 304 bp of plum DNA upstream and 591 bp downstream of the inverted repeat. Transgenic plum plants containing single or multiple copies of these hp inserts were inoculated with PPV D isolated from Pennsylvania, USA. PPV infection was evaluated through three cycles of coldinduced dormancy (CID) by symptom expression and by at least two and up to five ELISA and PCR tests. Of 24 plants evaluated nine were never infected, six in some tests showed weak infection, and nine plants were consistently infected. Most of the resistant lines contained a single copy of the minimal hp construct. These data strongly suggest that the hp portion of the PPV-CP insert in 'HoneySweet' plum is responsible for PPV resistance.

The hypersensitivity resistance of European plum to Plum pox virus and its potential impact on the epidemiology of the virus

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Since the early report on the Sharka disease in 1935, breeding for Sharka (Plum pox) virus resistance is considered to be the most promising way of minimising the outstanding economic damage caused by the disease in stone fruit production. Several resistance breeding strategies are embarked by breeders throughout the world. Those used in European plum (*Prunus domestica*) breeding were compared concerning their potential use in both minimizing the economic losses and avoiding the dissemination of PPV in orchards and over long distances.

Varieties tolerant to PPV are able to prevent the decline of plum production in areas where PPV is prevalent. However, they contribute to the dissemination of PPV into regions up to know free from PPV via latently infected plant material. For a long time, breeding for PPV resistance has been limited due to the lack of sources of resistance. Up to know, two different types of natural resistance have been found in *P. domestica*: The so called quantitative resistance and the hypersensitivity resistance. The latter inhibits the viral replication and dissemination within the plant. Hypersensitive genotypes are, under natural inoculation conditions, no source of PPV. Therefore, they do not contribute to the dissemination of PPV both via insect vectors within an orchard or via infected plant material. On the contrary, quantitatively resistant genotypes, even if they have low virus titer after inoculation with PPV, are sources of infection and hold the same disadvantages than tolerant varieties concerning the spread of PPV. It was shown that the hypersensitivity trait can be found in interspecific hybrids between *P. domestica* and $^{P.\ salicina}$ and between *P. domestica* and *P. cerasifera*. These genotypes can be used as rootstocks. The use of hypersensitive genotypes as rootstocks for Prunus species opens a new dimension in avoiding the dissemination of PPV via latently infected plant material.

Transient expression as a method to evaluate effectiveness of SCFV fragments to interfere with plum pox virus infection

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The constitutive expression of single chain Fv fragments (scFv) specific to different viral proteins in transgenic plants has been used to immunomodulate or interfere with viral infection. However in most of the referenced reports the level of protection was only partial. Probably the success of this approach relies on efficient scFv expression and accumulation and on the election of a relevant target protein. Transient expression mediated by agroinfiltration is an alternative method to the stable transformation to evaluate the stability and accumulation level of scFv fragments in the plant cell. Co-agroinfiltration experiments were carried out in Nicotiana benthamiana. Plants were agroinfiltrated with a co-cultures of recombinant Agrobacterium tumefaciens (carrying different versions of the scFv2A fragment-specific for the NIb replicase or the scFv5B fragment-CP specific) and an infective PPV-GFP clone. Agroinfiltrated leaves were excised after three days post-agroinfiltration, observed under stereomicroscope with UV light and photographed. A significant reduction on the number and size of the fluorescent foci were

observed with both scFv fragments and viral accumulation (estimated by DASI-ELISA) was also reduced.

The use of PPV-GFP allowed viral infection monitoring at early stages, and supplied valuable information about the immunomodulation (interference effects) produced by both scFv fragments against structural and non-structural PPV proteins.

Natural deletion is not unique in the coat protein (CP) of recombinant Plum pox virus (PPV) isolates in Hungary

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Plum pox virus (PPV) is the economically most important viral pathogen of stone fruit trees in Europe. PPV isolates characterized by different serological, molecular and epidemiological properties are grouped into three major (PPV-D, PPV-M, PPV-Rec) and three minor (PPV-EA, PPV-C, PPV-W) groups. During our study apricot (Prunus armeniaca L.) leaves showing typical symptoms of PPV infection were collected from Hungary in 2008. Samples were tested for the presence of PPV by RT-PCR using a Potyvirus-specific primers (PolyT2 and Poty7941), which amplified the 3'NIb–CP–3'UTR region form the viral genome. The PCR fragments were sequenced, and then sequence comparisons and phylogenetic analysis were done.

The multiple alignments of the nucleotide and deduced amino acid sequences corresponding to the 3'NIb-CP-3'UTR region showed that the isolate PPVGodollo2 bearing a 33-nucleotid (11-amino acid) natural deletion in frame in the N-terminal part of the coat protein (CP), downstream to the DAG amino acid motif. Currently we have reported on a Hungarian PPV-Rec isolate (PPV-B1298) collected from plum bearing a much larger, 135-nucleotide (45-amino acid) natural deletion at similar position to that of the PPV-Godollo2 (Szathmáry et al., Archives of Virology 154: 141–145, 2009).

According to the phylogenetic analysis of the nucleotide sequence of the 3'NIb– 5'CP region PPV-Godollo2 isolate clustered together with the previously characterized PPV-Rec isolates.

Our present study and previous data suggest that presence of natural deletion in the N-terminal part of the CP in the Hungarian recombinant PPV population is not unique.

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Symptomatic and real-time PCR scoring of Plum Pox Virus resistance in two apricot (*Prunus armeniaca* L.) segregating populations

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Resistance to Sharka disease, caused by the *Plum Pox Virus* (PPV), is one of the main aims in all the apricot breeding programmes currently in progress. Nevertheless, PPV resistance screening means a bottleneck for the programmes since it is very time consuming and requires a large greenhouse space. To date our lab performed the evaluation according to a biological test confirming the virus presence by ELISA-DASI. This procedure, although trustworthy, only allowed to classify seedlings into resistant (1) or susceptible (0). To get a more detailed picture of PPV resistance in segregating populations, we have follow the same biological test but implementing a symptomatic evaluation in which all leaves were scored individually. In addition, real-time PCR was performed on symptomatic and asymptomatic leaf samples to increase PPV detection sensitivity. For this purpose, two families, with different PPV resistance sources, have been evaluated, an F1 (`Goldrich' x 'Currot') and an F2 ('Lito' x 'Lito'). Results from this new scoring might be helpful to discern among the different hypothesis about the genetic control of apricot PPV resistance already reported.

Serological and molecular screening of Plum pox virus in the F1 and F2 romanian apricot genotypes

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Sharka (plum pox) is considered one of the most devastating diseases of stone fruits in terms of agronomic impact and economic importance (Dunez & Sutic, 1988; Nemeth, 1994). The disease is very detrimental in apricot, peach and plum trees because it produces reduced quality and premature dropping of fruits. The dynamics of virus infection in F1 and F2 romanian apricot cultivars (Prunus armeniaca) were analyzed in the natural infection conditions. The F1 and F2 romanian apricot progenies were serologically tested by DAS-ELISA using PPV-D and PPV-M specific monoclonal antibodies. Molecular tests were done by RT-PCR using P1/P2 primers (Wetzel et all., 1991). PPV detection by conventional serological and molecular methods was performed during the dormant period (wintertime). This period is important drawbacks due to false negative (sensivity) but also false positive (specificity). Romanian apricot F1 and F2 progenis evaluated were initially classified into three groups; susceptible to PPV, partially resistant to PPV and resistant to PPV. After that the most important genotypes were grafted onto infected GF305 in greenhouse conditions. The goals of the work presented in this communication are to introduce and to develop the marker-assisted selection (MAS) in PPV resistances F1 and F2 progenis in apricot Romanian cultivars.

Tracking Plum pox virus in Chile throughout the year by three different methods and molecular characterization of Chilean isolates

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During 2007 a survey was performed to detect and identify Plum pox virus (PPV) in Chilean stone fruits commercial orchards. Using primers P4b and P3D described by Candresse et al. (1998, Phytopathology 88,198-204) a fragment of 467 bp was amplified corresponding to the replicase-coat protein (Nib-CP) region from the virus genome. The fragment sequences obtained enabled to characterize all isolates as PPV-D, confirming that this is the only serotype present in Chile. To optimize the virus detection, 27 PPV-positive trees were selected, and sampled monthly from December 2006 until December 2007, collecting plant tissues available at the time of sampling (leaves, cuttings, buds and flowers). Each sample was analyzed by three different techniques: ELISA, RT-PCR and non-isotopic molecular hybridization (MH). For ELISA, the kit "Realisa Reforzado" (REAL, Spain) was used. The RT-PCR was carried out by using PPV universal primers P1/P2, whereas the probe used for HM corresponded to the CP region. The results showed that RT-PCR was more sensitive for detection in all months excepting January 2007, when the three techniques showed the same sensitivity. In general, MH showed a better sensitivity compared with ELISA. The best plant materials for analysis were: leaves, in February, March, September and October; phloem from cuttings in June; buds in July, and flowers in August. These results suggested that the use of RT-PCR should be considered for routine control of PPV in Chile, taking into account two major sampling windows during the year: the first, from July to September; and the second with slightly lower sensitivity, during the months of February and March. MH is shown as a clear alternative to the serological test for large-scale surveys.

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