

## Differentially expressed genes in healthy and plum pox virus-infected *Nicotiana benthamiana* plants

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Received June 23, 2015; revised July 22, 2015; accepted October 15, 2015

**Summary.** – Viruses use both material and energy sources of their hosts and redirect the production of disposable compounds in order to make viral replication more efficient. Metabolism of infected organisms is modified by these enhanced requirements as well by their own defense response. Resulting complex story consists of many regulation events on various gene expression levels. Elucidating these processes may contribute to the knowledge on virus-host interactions and to evolving new antiviral strategies. In our work we applied a subtractive cloning technique to compare the transcriptomes of healthy and plum pox virus (PPV)-infected *Nicotiana benthamiana* plants. Several genes were found to be induced or repressed by the PPV infection. The induced genes were mainly related to general stress response or photosynthesis, several repressed genes could be connected with growth defects evoked by the infection. Interestingly, some genes usually up-regulated by fungal or bacterial infection were found repressed in PPV-infected plants. Potential involvement of particular differently expressed genes in the process of PPV infection is discussed.

**Keywords:** virus-host interaction; gene expression; subtractive hybridization

### Introduction

Viruses are traditionally connected with diseases. It results from the fact that pathological changes enabled the discovery of their etiological agents and are logically in the center of interest concerning human, animal or plant health. Currently, modern laboratory tools enabled to discover that the constitution of living organisms is much more complex,

including plenty of cellular or subcellular species, as well as adapted and horizontally migrating genes in the frame of each multicellular body. Although many such intercellular „passengers“ contribute potentially to the evolution of their „vehicle“, from the practical point of view pathogenic organisms remain more important in the short term. Comparative studies on various molecular levels (transcripts, proteins) may help to better understand the relations between pathogens and their hosts (Haft, 2015).

*N. benthamiana* is a model species widely used in plant virology because of its sensitivity to a broad spectrum of viruses, usually systemic infection and easy maintenance (Goodin *et al.*, 2008). It is a proper propagative host also for viruses naturally infecting perennial plants, such as plum pox virus (PPV). We have extensively used this artificial host plant species, e. g. for multiplication of emerging PPV strains, as primary target for developing biolistic technique, for analysis of PPV strain competitiveness and of mutations accumulated in the PPV genome, or for expression of foreign genes cloned in the PPV genome (Glasa *et al.*, 2004; 2010; Predajňa *et al.*, 2010; Šubr *et al.*, 2012; Kamencayová

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**Abbreviations:** APX = ascorbate peroxidase; CAB = chlorophyll a/b-binding protein; DSPP = dentin sialophosphoprotein; FRL = Frigida-like protein; GCS = glycine cleavage system; HMGB = high mobility group B protein; JA = jasmonic acid; MLP = major latex protein; MT = metallothionein; PI-PLC = phosphoinositide-specific phospholipase C; PPV = plum pox virus; PRG = photosynthesis-related gene; PS II = photosystem II; ROS = reactive oxygen species; SNS = S-norcochlorogenic acid synthase; SUMO = small ubiquitin-related modifier; TLP = tetraspanin-like protein; Ub = ubiquitin

*et al.*, 2014). Here we attempt to compare the transcripts from healthy and PPV-infected *N. benthamiana* plants to reveal the regulatory effects in the course of systemic viral infection.

### Material and Methods

*In vitro* cultivated *N. benthamiana* lines were established from plants infected by PPV-Rec (isolate BOR-3) (Glasa *et al.*, 2004) and from healthy plants. Two hundred mg of leaf tissue from each line were used for total RNA isolation by Agilent Plant RNA Isolation Mini Kit (Agilent Technologies). In-Fusion SMARTer Directional cDNA Library Construction Kit (Clontech) was applied to prepare double stranded cDNA, which was subjected to subtractive hybridization exactly as described (Šubr *et al.*, 2002). Each sample (healthy and infected) was analysed both as the driver (digested by *AluI*) and the tracer (digested by *EcoRI*). Reconstituted fragments were cloned to *EcoRI*-digested pBluescript SK II, transformed into *E. coli* JM109 and obtained clones were analysed by colony PCR using insert-spanning primers. The amplicons were sequenced and subjected to BLAST analysis.

### Results and Discussion

Typically, tens to thousands clones harboring differentially expressed genes are obtained by subtractive hybridization techniques. In our experiment, 217 and 12 clones represented genes induced and repressed by PPV infection, respectively. The length of inserts ranged from 250 to 1500 bp. Only clones containing inserts longer than 750 bp from the first set and all twelve clones from the second set were selected for sequence analysis. Obtained results are summarized in the Table 1. Influenced genes coded for proteins implemented in various biochemical reactions. Causal connection with general stress or pathogen attack could be relatively clearly outlined for most of them. Several up-regulated genes were directly or indirectly connected with reactive oxygen species (ROS) production or relevant signal transduction. Short description of potential involvement of induced genes in plant response to virus infection follows.

Plant metallothioneins (MTs) are small cystein-rich proteins involved in binding of mono- and divalent metal ions, essential for metal homeostasis and detoxification (Leszczyszyn *et al.*, 2013). Induction of MT production has been observed in various plants after infection by bacteria, phytoplasmas or fungi. Connection of MT expression with different stress conditions including virus infections of plants has been recorded, particularly when senescence-like symptoms occurred (Choi *et al.*, 1996; Kim *et al.*, 2001). Rather than to direct interaction with pathogens, it may be related to the general „out-of-balance“ level of particular

molecular compounds resulting from the infection and the tendency of the host to renew its cellular homeostasis. The mechanism of MT action may be also related to antioxidative properties of its thiol groups (Carginale *et al.*, 2004). Under stress conditions namely, enhanced concentration of ROS can cause significant cell damage. During microbial infection a transient accumulation of H<sub>2</sub>O<sub>2</sub> causing localized programmed cell death leads to preventing further pathogen spread in the plant. Antioxidative compounds protect other host tissues from this action.

Another protein found in our work to be up-regulated during virus infection, ascorbate peroxidase (APX), has also a role in the protection against oxidative stress by ROS detoxification. It is the key enzyme of the ascorbate-glutathione cycle catalyzing the conversion of H<sub>2</sub>O<sub>2</sub> into water, using ascorbate as the donor of electrons. It may be activated in chloroplasts as well as in cytoplasm, mitochondria or peroxisomes (Caverzan *et al.*, 2012). Only weak constitutive expression of rice APX has been recorded under standard conditions, while strong transcriptional up-regulation occurred upon various stress events including wounding or fungal pathogen attack (Agrawal *et al.*, 2003). In another case, the grapevine APX was 3-times more accumulated in *Botrytis cinerea*-infected tissues, however, without enhancement of its transcription (Dadakova *et al.*, 2015). Enhanced APX activity has been found also in virus-infected plants (e. g. citrus tristeza virus in Mexican lime; Pérez-Clemente *et al.*, 2015). Interestingly, APX activity was induced by PPV infection only in a susceptible (contrary to a resistant) apricot cultivar (Hernández *et al.*, 2001).

Several nuclear genes coding for proteins active in the photosynthetic process (photosynthesis-related genes, PRGs) were found to be induced by the viral infection, namely chloroplast ferredoxin, photosystem II (PS II) subunit P2 and PS II reaction center W protein. Ferredoxin attends in the electron transport from photosystem I and in partitioning electrons to various enzymes (Fukuyama, 2004). Both PS II factors are required for assembly/stabilisation of the PS II complex. The concentration and/or activity of PRG-coded proteins use to be rather decreased in plant tissues during pathogen attacks (Bolton, 2009). In symptomatic chlorotic tissues of virus-infected plants the PRGs are down-regulated. As reported before, when light-dark mosaic symptoms occurred, photosynthetic apparatus was damaged only in chlorotic areas, the dark islands retained intact chloroplast structure and function (Chen *et al.*, 2015). PPV-Rec isolate BOR-3 used in our experiments induced only dark green areas around the main veins on *N. benthamiana* leaves and slight downrolling of the leaf blades. Such symptom expression is not in contradiction with estimated transcriptional induction of several PRGs. The P2 subunit may also play a role in ROS-dependent signaling as sensor for singlet oxygen generated in chloroplasts (Brzezowski *et al.*, 2012).

Table 1. Up- and down-regulated genes of *N. benthamiana* by PPV infection as determined by subtractive cloning

I/R*	Protein	BLAST hit details	
I	metallothionein	<i>Nicotiana tomentosiformis</i> MT-like protein type 2	XM_009608450.1
I	photosystem II subunit P2	<i>Nicotiana benthamiana</i> chloroplast PsbP2 precursor	JF897608.1
I	ascorbate peroxidase	<i>Nicotiana tomentosiformis</i> APX	XM_009608760.1
I	ferredoxin	<i>Nicotiana sylvestris</i> chloroplast ferredoxin	XP_009796636.1
I	photosystem II reaction center W protein	<i>Nicotiana sylvestris</i> PSII RCW	XM_009799309.1
I	major latex protein	<i>Nicotiana tomentosiformis</i> kirola-like	XM_009613937.1
I	transcription factor HY5	<i>Nicotiana sylvestris</i> transcription factor HY5	XM_009770015.1
I	mitochondrial ribosomal protein	<i>Nicotiana sylvestris</i> 54S ribosomal protein L19, mitochondrial-like	XM_009781628.1
I	ubiquitin-conjugating enzyme E2	<i>Nicotiana sylvestris</i> UCE E2 2	XM_009791022.1
I	phosphoinositide phospholipase C	<i>Nicotiana sylvestris</i> PI-PLC 6-like	XM_009785457.1
I	FRIGIDA-like protein	<i>Nicotiana sylvestris</i> FRL 4a	XM_009767899.1
I	dentin sialophosphoprotein-like	<i>Nicotiana sylvestris</i> DSPP	XM_009785372.1
R	defensin	<i>Nicotiana excelsior</i> $\gamma$ -thionin	AB005266.1
R	protease inhibitor	<i>Nicotiana tomentosiformis</i> proteinase inhibitor I-B-like	XM_009607371.1
R	S-noroclaurine synthase	<i>Nicotiana sylvestris</i> SNS-like	XM_009768642.1
R	chlorophyll a/b-binding protein	<i>Nicotiana sylvestris</i> CAB	AB012637.1
R	deSUMOylating isopeptidase	<i>Nicotiana tomentosiformis</i> deSUMO IP2-like	XM_009630754.1
R	glycine cleavage system H protein	<i>Nicotiana tomentosiformis</i> GCSH, mitochondrial-like	XM_009625854.1
R	$\beta$ -galactosidase	<i>Nicotiana tomentosiformis</i> $\beta$ -galactosidase	XM_009629241.1
R	high mobility protein B2	<i>Nicotiana tabacum</i> HMGB2-like	EF051129.1
R	tetraspanin-like protein	<i>Nicotiana sylvestris</i> TLP	XM_009798164.1
R	chloroplast protein CP12	<i>Nicotiana tabacum</i> CP12	AF359459.2
R	unknown	<i>Nicotiana tomentosiformis</i> uncharacterized LOC104084708	XM_009588631.1
R	unknown	<i>Nicotiana sylvestris</i> uncharacterized LOC104214887	XM_009764610.1

\*I = induction, R = repression.

Kirola, the kiwifruit allergen belongs to the family of major latex proteins (MLPs) according to the sequence similarity, as do other kirola-like proteins from other species. X-ray diffraction and immunological studies showed their close structural relationship to pathogenesis-related proteins of the class PR-10 with a small but highly hydrophobic ligand-binding cavity (Chruszcz *et al.*, 2013). Evidence for the MLP involvement in defensive responses to biotic and abiotic stresses has been demonstrated. MLP was abundant in both cotton root and hypocotyl tissues infected with *Fusarium oxysporum* (Dowd *et al.*, 2004). Although its real function is unknown, MLP overexpression during fungal and viral infection, salt and drought stress, or toxin exposure has been confirmed (Chen and Dai, 2010; Malter and Wolf, 2011).

LONG HYPOCOTYL5 (HY5), a basic domain/leucine zipper protein, is the pivotal transcription factor for photomorphogenesis that is regulated by multiple photoreceptors. HY5 binds to the promoters of a large number of genes that have diverse functions in plant growth and development including photosynthetic machinery assembly, photopigment production, chloroplast development, and seedling cotyledon expansion. Many of the regulated genes code also for transcription factors, often regulating expression of PRGs or circadian oscillators, so HY5 is probably a higher

hierarchical regulator of the transcriptional cascade for photomorphogenesis (Lee *et al.*, 2007). Induction of HY5 during PPV infection thus correlated with enhanced transcription levels of PRGs mentioned above.

A rise in respiration rate has been often observed in plant viral infections. The energy is needed both for virus replication and plant defense reactions, thus the infection is a highly energy-demanding process (Bolton, 2009). Key enzymes for oxidative phosphorylation are coded by mitochondrial DNA and expressed by mitochondrial proteosynthetic apparatus. Therefore, an enhanced production of mitochondrial ribosome compounds during viral infection is not surprising.

The ubiquitin (Ub)/26S proteasome system is the main proteolytic pathway in eukaryotes, which helps to remove abnormal proteins and regulates many cellular processes by targeted proteolysis of functional polypeptides. As such a general mechanism, ubiquitination is directly or indirectly involved in each biological process in plants and also plays a role in disease resistance signaling. It has also been shown usurped by several viruses to regulate their own replication in the host cell (Alcaide-Loridan and Jupin, 2012; Choi *et al.*, 2013). The Ub-conjugating enzymes E2 are mediators of final Ub binding to proteins by E3 (Ub-ligating enzymes). E2 induction by heat or cadmium chloride treatment in tomato

has been demonstrated (Feussner *et al.*, 1997). Overexpression of E2 in transgenic *A. thaliana* enhanced the tolerance of drought stress (Wan *et al.*, 2011). On the other hand, cotton leaf curl Multan geminivirus infection interfered with E2 and inhibited ubiquitination *in planta* (Eini *et al.*, 2009).

Phosphoinositide-specific phospholipase C (PI-PLC) is an enzyme catalysing hydrolysis of phosphatidylinositol 4,5-bisphosphate into the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. It has been shown to be associated with signal transduction during various types of stress conditions including salt, drought and osmotic stress, as well as pathogen response (Rupwate and Rajasekharan, 2012). Although the mechanism is unclear, it may be indirectly connected with known PI-PLC influence on guard cell action related to stomatal regulation of gas flow (Mills *et al.*, 2004). Dardick (2007) found PI-PLC rather repressed by PPV infection of *N. benthamiana*. This discrepancy may reflect different experimental conditions of these experiments (infection stage, virus isolate) and indicates various functions of PI-PLC in complex virus-host interactions.

Frigida of *A. thaliana* is known as an essential factor for regulation of flowering time. It prevents too early flowering by induction of the floral repressor through a cotranscriptional mechanism involving interaction with the nuclear cap-binding complex. However, several FRI families have been identified, which probably have separate biological roles (Risk *et al.*, 2010). Nonfunctional FRI alleles had negative pleiotropic effect on plant fitness by reducing the numbers of nodes and branches on the inflorescence (Scarcelli *et al.*, 2007). The role of several detected Frigida-like proteins (FRLs) in this process is unknown, although they are believed to be required, together with FRI, for the winter-annual habit. There is no evidence for their involvement in any pathogen- or stress-related process and no significant delay in flowering of virus-infected plants was observed.

Dentin sialophosphoprotein (DSPP) is the precursor of two non-collagenous compounds of dentin (and to some extent of bone) tissues: dentin sialoprotein and dentin phosphoprotein, involved in dentinogenesis (Qin *et al.*, 2002). Plant analogues of DSPP may take part e.g. in biomineralisation process at phytolith production. They have been transcriptionally induced in wheat infected by *Fusarium graminearum*, and involved in arsenic-tolerance of *A. thaliana*, so its function in plant metabolism under stress conditions cannot be excluded (Hill-Ambroz *et al.*, 2006; Fu *et al.*, 2014b).

Some genes influencing growth and cell division were repressed by PPV infection. Interestingly, several down-regulated genes also coded for proteins participating in interactions with plant (mostly cellular) pathogens. It may reflect the dominance of the virus in some but not all „battles“ of the molecular „war“.

Plant defensins are small, cysteine-rich peptides that constitute a part of the innate immune system primarily

directed against fungal pathogens. They are relatively abundant in seed coat, where during germination, they protect the seedlings from soil fungi. Defensins have been identified also in other tissues, mainly in peripheral cells and stomatal cells (Stotz *et al.*, 2009). Pathogen- and other environmental stress-inducibility of defensins has been reported. Signaling pathways controlling these plant responses to microbial infection operate through the action of phytohormones like jasmonic acid (JA). During viral infection, however, JA-regulated gene expression is rather suppressed by RNA silencing (Hanks *et al.*, 2005; Westwood *et al.*, 2014). As a consequence, virus-infected plants may be more susceptible to secondary bacterial or fungal infections.

Plant protease inhibitors induced by wounding or microbial attacks are capable of suppressing enzymatic activity of phytopathogenic microorganisms or saliva of feeding insects (Ryan, 1990). Cysteine proteases and their inhibitors are probably regulation elements of programmed cell death in plants (Solomon *et al.*, 1999). Although some plant viruses (including potyviruses) use their own specific proteases in the genome expression strategy, there is no evidence of specific antiviral response by active protease inhibition. Down-regulated genes for protease inhibitors (similar to the defensin gene) in our experiment may reflect the complex situation, when some anti-fungal/anti-bacterial defense mechanisms are rather repressed, possibly to the detriment of anti-viral pathways.

S-norcoclaurine synthase (SNS) belongs to the group of pathogenesis-related proteins PR-10 participating in the defense of plants against microorganisms and fungi (Lee and Facchini, 2010). Its transcriptional repression fits the aforementioned picture. Transcription level of SNS declined also after opium poppy treatment by methyl jasmonate (Gurkok *et al.*, 2014). It may indicate its negative regulation by JA-based defense reaction.

The nuclear gene for chlorophyll a/b-binding protein (CAB), a key photosynthesis factor, has been shown induced under drought stress conditions in faba bean (Abid *et al.*, 2015). However, in most cases it was down-regulated in abiotic or biotic stress-suffered plants including transgenic tobacco plants expressing the full-length genome of tobacco mosaic virus (Jada *et al.*, 2014). Experiments with retrograde signaling pathway mutant *A. thaliana*, where the CAB expression is uncoupled from the functional state of chloroplast, demonstrated that plastid signals controlling the expression of nuclear genes are necessary for defense responses to viral infection (Fu *et al.*, 2014a).

DeSUMOylating isopeptidase IP2 is a C97 class cysteine protease involved in Small ubiquitin-related modifier (SUMO)-mediated protein degradation. SUMOylation and deSUMOylation is a general post-translational regulatory mechanism of biological processes in plants including hormonal responses, flowering or defense against various

types of stress. Disturbance of SUMO homeostasis causes abnormal control of the plant defense response: SUMO proteases may promote pathogen multiplication and delay the host response (Miura and Hasegawa, 2010). For example, SUMOylation of potyviral replicase has been shown to play a crucial role in the establishment of the infection (Xiong and Wang, 2013). Therefore, inhibition or repression of SUMO proteases can be regarded as manifestation of active plant defense.

Glycine cleavage system (GCS) is an enzyme complex bound to the inner mitochondrial membrane, taking part in glycine decarboxylation during respiration. The H protein (GCSH) with bound lipoic acid functions as a shuttle for some intermediate reaction products and interacts with other enzymes of the system. Involvement of GCS in plant defense response has been presumed, as its inhibition induced the production of ROS (Palmieri *et al.*, 2010). GCSH level decreased in *A. thaliana* infected by *Botrytis cinerea* (Mulema *et al.*, 2013). In cold- and drought-stressed pea plants, the loss of mitochondrial glycine oxidation capacity was correlated with GCSH delipoidation (Taylor *et al.*, 2002).

$\beta$ -galactosidases are glycosyl hydrolases commonly associated with the hydrolysis of lactose by mammals and *E. coli*. They also belong to the set of cell wall-degrading enzymes expressed by plant pathogenic fungi.  $\beta$ -galactosidase can release stored energy for rapid growth (xyloglucan mobilization in cotyledons), act at metabolic recycling of galactolipids and glycoproteins, or degrade cell wall components during senescence. It has been shown important for fruit ripening (Smith and Gross, 2000). However, scarce data are available about possible role of  $\beta$ -galactosidase in plant-pathogen interactions. Involvement of interactions between primary and secondary metabolism pathways cannot be excluded. Flavonoids are secondary metabolites involved in general stress tolerance of plants. They accumulate *in vivo* in form of glycosides, and a direct link between the production of flavonoids and sugars has been suggested (Pourcel *et al.*, 2007). Significant negative correlation of  $\beta$ -galactosidase and flavonoid levels has been estimated by a study of cold stressed white clover (Rasmussen *et al.*, 2006). Down-regulation of  $\beta$ -galactosidase in pathogen-infected plants may increase the concentration of flavonoids, which act as scavengers of ROS.

Chromosomal high mobility group B proteins (HMGB) are characterized by a HMG-box DNA-binding domain flanked by a basic N-terminal and an acidic C-terminal domain. These non-histone chromatin-binding proteins act as versatile modulators of DNA function including influence on transcription and recombination (Pedersen and Grasser, 2010). The expression of HMGB genes was shown differentially regulated by stress and vice versa, plants overexpressing or lacking certain HMGB proteins displayed altered responses to stress treatment. Overexpression of

HMGB2 reduced *Arabidopsis* seed germination under salt and drought stress (Kwak *et al.*, 2007). Its role in adaptation of plants to changing environmental conditions has been confirmed also with cold-suffering seabuckthorn (Ghangel *et al.*, 2012). While HMGB2 has been induced by poliovirus infection in an animal model (Grinde *et al.*, 2007), this is the first demonstration of its relation to a plant virus infection.

Animal tetraspanins are proteins with four transmembrane domains involved in intercellular communication with diverse functions in cell motility, proliferation, differentiation, cell fusion and signaling. Their fungal homologs (tetraspanin-like proteins, TLPs) are essential for penetration of fungal pathogens into leaves of the host plants (Clergeot *et al.*, 2001; Gourgues *et al.*, 2004). Function of structurally similar plant TLPs is still unknown, their involvement in male gamete-related processes during pollination has been hypothesized. Plant TLPs are essential for normal plant development, mutations of TLP gene resulted in severe pleiotropic defects in morphology affecting the shoot apical meristem (Wang *et al.*, 2012). Thus, inhibition of TLP expression by viral infection may cause e.g. deformations, especially in evolving apical leaves due to faulty regulation of cell-growth and cell-positioning. Primary function of such inhibition may be repression of TLP-related signal transduction.

Chloroplast protein CP12 is a small, intrinsically disordered redox-sensitive protein expressed in a range of tissues including roots, flowers, and seeds, which influences carbohydrate partitioning. It has been hypothesized that CP12 acts to regulate metabolic pathways in response to changes in redox status in the chloroplast network. In higher plants its deficiency has a dramatic impact on the growth and morphology (loss of apical dominance, fused cotyledons, altered leaf morphology). CP12 has been shown to protect two enzymes of the Calvin-Benson cycle against oxidative stress (López-Calcagno *et al.*, 2014). Moreover, transcriptome analysis of CP12-suppressed tobacco plants *inter alia* revealed induction of photosystem II reaction center W protein and metallothionein-like protein (both detected in our study), which may indicate functional connectivity between them in the frame of stress-related network (Howard *et al.*, 2011).

Two of the repressed transcripts were assigned as „uncharacterized“, however, one of them has been annotated as potential nucleoside-diphosphate kinase with a Pleckstrin homology domain. This domain binds phosphatidic acid, an early signal in the defense response.

We showed that despite some limits in effectivity, the simple subtraction cloning protocol leads to relevant results for comparison of differentially expressed genes on the transcriptional level. Low-range experiments of this type may validate and supplement the data obtained from extensive transcriptomic studies (Whitham *et al.*, 2003).

All our BLAST results referred to *Nicotiana* spp. and most of detected genes (with exception of FRL) have been connected with stress-related metabolism before. Induction of genes related to ROS production and signal transduction, and up-regulated JA-dependent pathways correlated well with known antiviral defense processes. Other pathways directed mainly against fungal or bacterial pathogens were rather down-regulated, and some repressed genes could be connected with manifestation of macroscopic symptoms of viral infection.

Comparative transcriptomics shows only the differences in gene expression but it cannot elucidate the causal connections and mechanisms of interactions leading to the status quo. Complex picture involves direct viral usurpation of cellular machinery, host defense reactions, as well as virus-induced repression of these reactions. In natural compatible virus-host systems the interactions reflect long-term coevolution of both entities.

The stone fruit trees (*Prunus* spp.) are natural hosts of PPV. Although systemically infected, the trees show notable variation of virus and symptom distribution, in particular in sprouts and leaves (Šubr and Glasa, 2008). Analysis of peach (*P. persica*) gene expression in response to PPV infection using massive cDNA sequencing showed different changes of expression profiles in symptomatic and non-symptomatic leaves compared to healthy control. In non-symptomatic leaves, three-times more genes have been found affected by PPV infection (huge majority of them up-regulated) than in leaves showing symptoms (with similar number of up- and down-regulated genes) (Rubio *et al.*, 2015). Only few hits corresponding to our results have been recorded, mainly in leaves without symptoms (ferredoxin and MLP; some genes were found regulated in opposite way – MT, CAB and  $\beta$ -galactosidase).

*Arabidopsis thaliana*, a widely used model species in plant genomics, has also been studied for transcriptome alteration by PPV infection (Babu *et al.*, 2008). PI-PLC and DSPP-like protein were the only hits of that work corresponding to our results. MT, ferredoxin and MLP were down-regulated,  $\beta$ -galactosidase and HMG proteins up-regulated (opposite to our finding) (Babu *et al.*, 2008).

Changes of *N. benthamiana* transcriptome caused by three different viruses including PPV have been analysed by microarray technology (Dardick, 2007). Significant overlap of influenced genes has been recorded, although their number correlated with severity of symptoms induced by respective viruses. Extensive supplementary data of that work involve seven genes corresponding to our results (induction of MT, APX, Ub-conjugating enzyme, DSPP-like protein, repression of proteinase inhibitors, CAB and  $\beta$ -galactosidase). Several SUMO proteins have been induced by PPV infection (Dardick, 2007), while deSUMOylating enzyme was shown to be down-regulated in our results, both indicating

involvement of SUMOylation in the PPV infection cycle. Three up-regulated genes from our work (ferredoxin, PS II reaction center W protein and 54S ribosomal protein L19) were shown influenced by Tomato ringspot virus but not by PPV (Dardick, 2007).

PPV-induced regulation of particular plant genes obviously depends on the host species. Best match of the transcriptome modifications we described was obtained with data from *N. benthamiana*. It is a non-natural host, not evolutionary adapted to PPV infection. However, there are several benefits of this artificial virus-host system (Goodin *et al.*, 2008). The infection is systemic and PPV is relatively uniformly spread in all host tissues few days after infection (contrary to *Prunus* spp. hosts). Thus the experiments are more reproducible and require much less time, the plant maintenance and sample preparation are simple and fast. Of course, obtained results do not necessarily reflect interactions of the virus with its natural host, however, they contribute to the general knowledge about infection-induced changes of gene expression in plants.

**Acknowledgements.** The research was supported by the grant 2/0001/15 from the Scientific Grant Agency of Ministry of Education and Slovak Academy of Sciences (VEGA).

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