Department of Eukaryote Gene Engineering

Research overview

Department of Eukaryote Gene Engineering is focused on the research directions of recombinant viral and human protein analysis, expression and assembly of proteins into virus-like particles (VLPs) as well as mechanisms of plant signaling and biosynthesis. We are using methods of gene cloning and expression in different host systems, electron microscopy, immunological and functional biochemical assays of proteins, biosynthesis and purification of essential amounts of target recombinant proteins with application area including diagnostics and therapeutics. Please find few examples of our research studies during 2013-2016.

Development of porcine circovirus type 2 diagnostics tools

Porcine circovirus type 2 (PCV2)-associated diseases are responsible for significant mortality among pigs and remains a serious economic problem to the swine industry worldwide leading to significant negative impacts on profitability of pork production. The two major groups designated as PCV2a, PCV2b are found worldwide and have been demonstrated to be virulent enough to trigger PCV2-associated diseases. We determined the genomes of PCV2 virus spread in Lithuania pig farms in the samples collected between 2009 and 2013 and confirmed that all isolated virus genomes belonged to the PCV2b genotipe. Three slightly different open reading frame 2 (ORF2) variants were amplified by PCR from native PCV2 genomes as well as ORF2 of one PCV2-Cap gene variant was codon-optimized by *S. cerevisiae* codon usage and used for the expression in yeast *S. cerevisiae*. Recombinant PCV2b capside protein generated in yeast assembled into particles (Cap VLPs) similar to native virions (Fig.1). Yeast-derived PCV2 Cap VLPs were capable to induce the generation of PCV2-specific MAbs that did not show any cross-reactivity with PCV1-infected cells. Moreover, yeast-derived recombinant PCV2 Cap VLPs were used as antigen in newly developed the indirect IgG PCV2 Cap VLP-based ELISA. The high sensitivity and specificity of newly developed the indirect IgG PCV2 Cap VLP-based ELISA clearly suggested that this assay is potentially useful diagnostic tool for screening PCV2-suspected samples [Nainys J et al, 2014]. The new two-step chromatographic purification procedure of PCV2 Cap VLPs from yeast lysate was developed using Q Sepharose XL and cation-exchange CIMmultus SO3 monolith [Zaveckas M et al, 2015]. This work was funded by European Social Fund, grant No VP1-3.1-ŠMM-10-V-02-017.

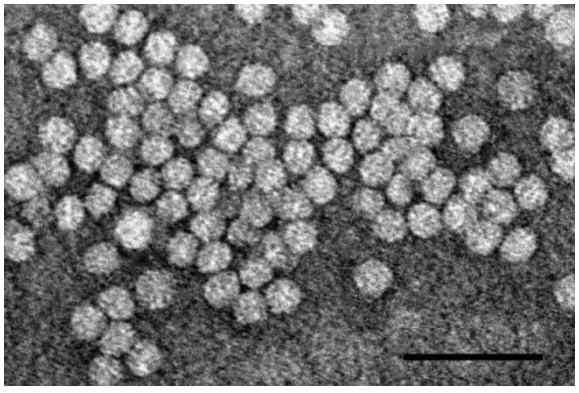


Fig.1. Electron microscopy pictures of VLPs formed by PCV2-Cap protein generated in yeast stained with 2% aqueous uranyl acetate solution and examined by Morgagni 268 electron microscope. Scale bar 100 nm.

Production of recombinant human polyomavirus-like particles in yeast

Over recent years eleven new human polyomaviruses (HPyVs) have been identified. According to preliminary serological studies all this variety of HPyVs subclinically infects the general population at an

early age. As many HPyVs cannot be easily cultured, major capsid protein VP1 is an ideal protein for generation of virus-like particles (VLPs) which resemble native virions they are derived from in structure, immunogenicity and tropism, but do not contain any viral genetic material. Recombinant VP1 VLPs originated from eleven newly identified HPyVs were efficiently produced in yeast [Norkiene M et al, 2015]. Merkel cell polyomavirus (MCPyV) and trichodysplasia spinulosa-associated polyomavirus (TSPyV) derived VP1 self-assembled into homogeneous in size VLPs. Karolinska Institute polyomavirus (KIPyV), HPyV7, HPyV9, HPyV10 and St. Louis polyomavirus (STLPyV) VP1 proteins formed heterogeneous in size VLPs with diameters ranging from 20 to 60 nm. Fractions of smaller VLPs (25–35 nm in diameter) dominated in preparations of VP1 proteins originated from Washington University polyomavirus (WUPyV) and HPyV6. HPyV12 VP1 VLPs were generated from the second of two potential translation initiation sites in VP1-encoding open reading frame. Yeast-produced recombinant VP1 VLPs originated from different HPyV demonstrated distinct HA activity and could be useful in virus diagnostics and capsid structure studies or investigation of entry pathways and cell tropism of new HPyVs replacing corresponding viruses until cell culture systems for the new HPyVs will be developed (Fig.2). This work was funded by the European Social Fund under National Integrated Program Biotechnology & Biopharmacy, grant No. VP1-3.1-SMM- 08-K01-005.

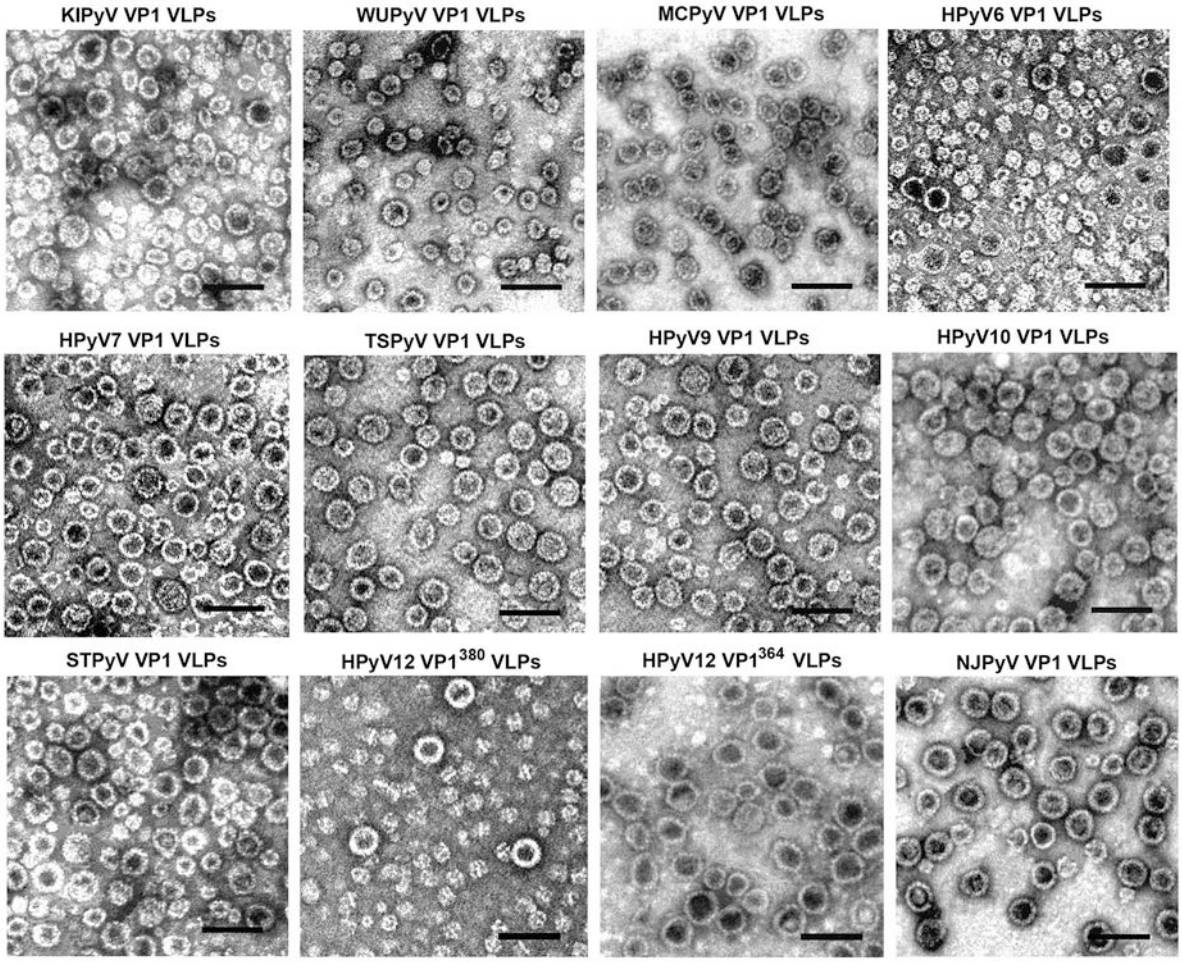


Fig.2. Electron microscopy pictures of VLPs formed by novel human polioma viruses-derived VP1 VLPs generated in yeast stained with 2% aqueous uranyl acetate solution and examined by Morgagni 268 electron microscope. Scal bar 100 nm.

Polyomavirus-derived virus-like particles as universal carriers of foreign peptides and single chain antibodies

The hamster polyomavirus (HaPyV) major capsid protein VP1 VLPs have been used as carriers for a variety of different foreign peptides, protein segments and entire proteins of different origin including virus- and cancer associated. A major reason for generation and using of chimeric VLPs is to transfer the intrinsic strong immunogenicity of the VLP carrier to per se low immunogenic peptide sequences. HaPyV VP1-derived VLPs were further explored for generation of chimeric VLPs as tools for hybridoma technology. Our approach to use chimeric VLPs was proven useful for the generation of virus-reactive MAb against hantavirus Gc glycoprotein. The generated broadly-reactive MAb #10B8 might be useful for various diagnostic applications [Zvirbliene A et al, 2014].

The localization of potential insertion sites for foreign peptide sequences is essential for the generation of chimeric VLPs as well as evaluation of foreign peptide size limits that can be inserted. The evaluation of new carrier for construction of chimeric VLP - Trichodysplasia spinulosa-associated polyomavirus (TSPyV) VP1 VLPs - was exploited in comparison to hamster polyomavirus VP1 protein. The insertion sites were selected based on molecular models of TSPyV VP1 protein. Chimeric VLPs harbouring inserted either hepatitis B virus preS1 epitope DPAFR or a universal T cell-specific epitope AKFVAAWTLKAAA were capable to induce a strong immune response in mice, activated dendritic cells and T cells. This demonstrated that TSPyV VP1 protein represents a new potential carrier for construction of chimeric VLPs harboring target epitopes [Gedvilaite A et al, 2015].

HaPyV VP1/VP2 pseudotype VLPs was used as carrier for fused with VP2 protein a surface-exposed functionally active neutralizing antibody specific to hepatitis B virus (HBV) surface antigen (HBsAg). Formation of VLPs was confirmed by electron microscopy. The antigen-binding activity of the purified pseudotype VLPs was evaluated by ELISA and virus-neutralization assay on HBV-susceptible primary hepatocytes from Tupaia belangeri. The pseudotype VLPs were functionally active and showed a potent HBV-neutralizing activity comparable to that of the parental monoclonal antibody. Polyomavirus-derived pseudotype VLPs harbouring multiple functionally active antibody molecules with virus-neutralizing capability may represent a novel platform for developing therapeutic tools with a potential application for post-exposure or therapeutic treatment of viral infections [Pleckaityte M et al, 2015]. This research was funded by the European Social Fund under the Global Grant measure (Grant No. VPI-3.1-SMM-07-K-02-039).

Survey of molecular chaperone requirement for the biosynthesis of hamster polyomavirus VP1 protein in *Saccharomyces cerevisiae*

A number of viruses utilize molecular chaperones during various stages of their life cycle. It has been shown that members of the heat-shock protein 70 (Hsp70) chaperone family assist polyomavirus capsids during infection. However, the molecular chaperones that assist the formation of recombinant capsid viral protein 1 (VP1)-derived virus-like particles (VLPs) in yeast remain unclear. A panel of yeast strains with single chaperone gene deletions was used to evaluate the chaperones required for biosynthesis of recombinant hamster polyomavirus capsid protein VP1. The impact of deletion or mild over-expression of chaperone genes was determined in live cells by flow cytometry using enhanced green fluorescent protein (EGFP) fused with VP1. The results confirmed the participation of cytosolic Hsp70 chaperones and suggested the potential involvement of some Hsp40 co-chaperones and Hsp90 in the biosynthesis of VP1 VLPs in yeast [Valaviciute M et al, 2015]. This work was funded by the European Social Fund under the Global Grant Measure (Grant No. VP1-3.1-SMM-07-K-02-038).

Identification and characterization of polyomaviruses in wild voles and GASH:Sal Syrian golden hamsters suffering from lymphomas

Two novel polyomaviruses (PyVs) were identified in kidney and chest-cavity fluid samples of wild bank voles (*Myodes glareolus*) and common voles (*Microtus arvalis*) collected in Germany. All cloned and sequenced genomes had the typical PyV genome organization, including putative open reading frames for early regulatory proteins large T antigen and small T antigen on one strand and for structural late proteins (VP1, VP2 and VP3) on the other strand. Virus-like particles (VLPs) were generated by yeast expression of the VP1 protein of both PyVs. VLP-based ELISA and large T-antigen sequence-targeted polymerase chain reaction investigations demonstrated signs of infection of these novel PyVs in about 42% of bank voles and 18% of common voles. Phylogenetic and clustering analysis including all known PyV genomes placed novel bank vole and common vole PyVs amongst members of the tentative Wukipolymavirus genus. The finding of novel vole-borne PyVs may suggest an evolutionary origin of ancient wukipolyomaviruses in rodents and may offer the possibility to develop a vole-based animal model for human wukipolyomaviruses [Nainys J et al, 2015]. This work was funded by the European Social Fund under National Integrated Program Biotechnology & Biopharmacy, grant No. VP1-3.1-SMM- 08-K01-005.

Almost 16 % (90 males and 60 females) of the 975 GASH:Sal Syrian golden hamsters (Mesocricetus auratus) were affected during a 5-year period by the development of a progressing lymphoid tumour and exhibited similar clinical profiles, and a rapid disease progression resulting in mortality within 1 to 2 weeks. A TaqManprobe-based real-time PCR analysis of genomic DNA, immunohistochemical analysis using HaPyV-VP1-specific monoclonal antibodies, indirect ELISA and western blot analysis confirmed the presence of viral proteins in all hamster tumour tissues, the presence of antibodies against the VP1 capsid protein in sera. The HaPyV genome that accumulated in tumour tissues typically contained deletions affecting the noncoding regulatory region and adjacent sequences coding for the N-terminal part of the capsid protein VP2. The analysis of the non-deleted HaPyV genome derived from GASH-Sal hamster tumour tissues (JX416849) in comparison with the sequence of HaPyV isolated from Syrian hamsters in Berlin-Buch (JX036360) revealed 132 nucleotide exchanges (112 nucleotide exchanges were in the coding sequence, which resulted in a total of 23 amino acid exchanges in six viral proteins, suggesting that in the Salamanca new strain of HaPyV was responsible for the infection which did not originate directly from the same source as Berlin-Buch HaPyV strain [Munoz LJ et al, 2013].

Functional analysis of recombinant lipolytic protein encoded in phytoplasma phage based genomic island

Wall-less bacteria known as phytoplasmas are obligate transkingdom parasites and pathogens of plants and insect vectors. These unusual bacteria possess some of the smallest genomes known among pathogenic bacteria, and have never been successfully isolated in artificial culture. Disease symptoms induced by phytoplasmas in infected plants include abnormal growth and often severe yellowing of leaves, but mechanisms involved in phytoplasma parasitism and pathogenicity are little understood. A phage based genomic island (sequence variable mosaic, SVM) in the genome of Malaysian periwinkle yellows (MPY) phytoplasma harbors a gene encoding membrane-targeted proteins, including a putative phospholipase (PL), potentially important in pathogen-host interactions. The present study provided for the first time phytoplasma gene expression investigated and compared in two different protein expression systems: bacterial (*E.coli*) and yeast (*S.cerevisiae*). Despite the low level of expression of rPL in both hosts, a sufficient amount of the enzyme was purified for activity testing. The enzyme activity tests revealed that phytoplasmal rPL from MPY phytoplasma demonstrated only moderate PLA1and low PLA2 enzyme activity on substrates incorporated in liposomes and may be called a phospholipase with low PLA activity. The results obtained in the present study show the existence of an active lipolytic enzyme, encoded in the genomic SVM region of MPY phytoplasma [Gedvilaite A. et al., 2014].

Development of a diagnostic system which detects anti-Schmallenberg virus antibodies in bovine serum, saliva and milk

Schmallenberg virus (SBV), discovered in continental Europe in late 2011, causes mild clinical signs in adult ruminants, including diarrhoea and reduced milk yield. However, fetal infection can lead to severe malformation in newborn offspring. To develop improved reagents for SBV serology a high-level yeast expression system was employed to produce recombinant SBV nucleocapsid (N) proteins with and without hexa-histidine tag. Recombinant SBV N proteins were investigated as antigens in SBV-specific IgG enzyme immunoassay. Both yeast-expressed SBV N proteins were reactive with anti-SBV IgG-positive cow serum specimens collected from different farms of Lithuania (Fig.3). Additionally, N protein without His-tag reacted with milk and saliva samples of SBV seropositive cows. His-tagged N protein was used for generation of monoclonal antibodies (MAbs) in mice. Four MAbs raised against recombinant SBV N protein were generated and reacted with native viral nucleocapsids in SBV-infected BHK cells in immunofluorescence assay (Fig.4.). In summary, yeast-expressed SBV N proteins and newly developed SBV-reactive MAbs may provide useful reagents for diagnostics and seroprevalence studies of SBV infection. The study demonstrates that yeast expression system is suitable for high-level production of recombinant SBV N proteins and provides the evidence on the presence of SBV-positive antibodies in cow serum, milk and saliva specimens collected in Lithuania [Lazutka J. et al, 2014, Lazutka J. et al, 2015]. This work is supported by Lithuanian Science Council grant MIP-044/2013.

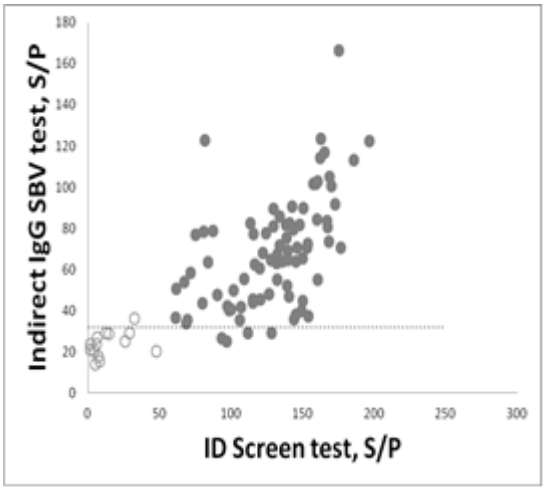


Fig. 3. Antibody responses of individual cow serum specimens defined by the newly developed indirect IgG SBV ELISA based on yeast-expressed SBV N protein in comparison to the commercial ID screen test. The S/P ratios of reactivity were plotted. Grey markers represent positive and white negative serum samples obtained by commercial ID screen test. The dotted line represents the cut-off value of the newly developed indirect IgG SBV ELISA.

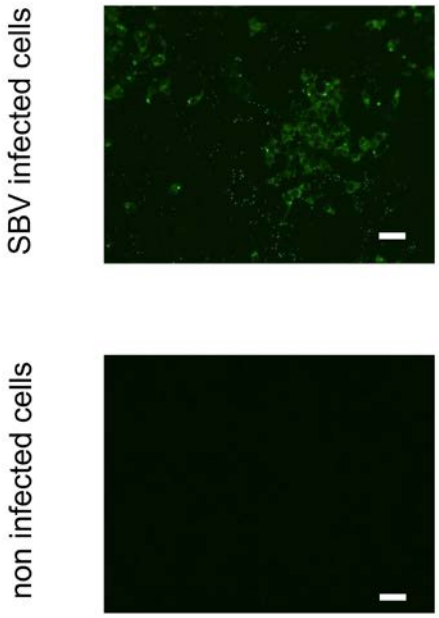


Fig. 4. Fluorescence microphotographs showing the reactivity of the MAbs 4F3 with BHK cells infected with SBV BH80/11 strain (upper). Noninfected BHK cells were used as a negative control ((lower). Hybridoma culture supernatants were used at a dilution of 1 : 10. Scale bar: 100 μm.

Study of nucleocapsid protein of human parainfluenza viruses 2 and 4

Human parainfluenza viruses 2 and 4 (HPIV2, HPIV4) cause respiratory tract infections. They belong to the genus *Rubulavirus* of the family *Paramyxoviridae*. The research aimed to produce nucleocapsid protein N of HPIV2 and HPIV4 in yeast *S. cerevisiae*. The HPIV2 N gene encoding amino acid sequence RefSeq NP_598401.1 was expressed but the protein did not form nucleocapsid-like particles (NLPs) characteristic to yeast-produced *Paramyxoviridae* N proteins. PCR mutagenesis was carried out to change the encoded specific aa residues to the ones conserved across HPIV2 isolates. The substitution NP_598401.1:p.D331V concluded in NLP formation (Fig. 5). Protein 3D-structure model (ModBase) suggested that this amino acid position was in the hydrophobic core of the C-terminal domain facing the inside of the domain. It is plausible that a negatively charged aspartic acid side chain in place of valine disrupts the packaging of α-helices, resulting in deformed monomers unable to multimerize correctly. Meanwhile, HPIV4 N protein successfully formed NLPs when expressed in yeast.

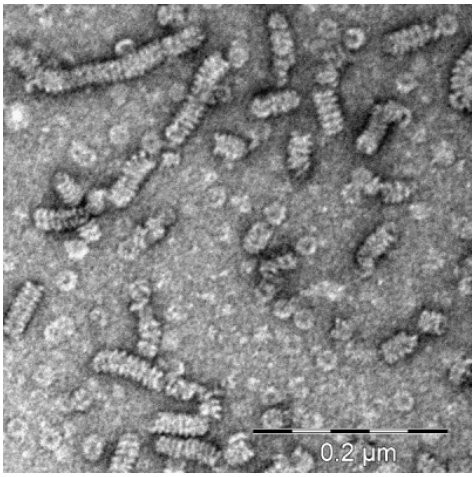


Fig. 5. Electron micrograph of CsCl density gradient purified HPIV2 N_{VRP} NLPs. Scale bar 200 nm.

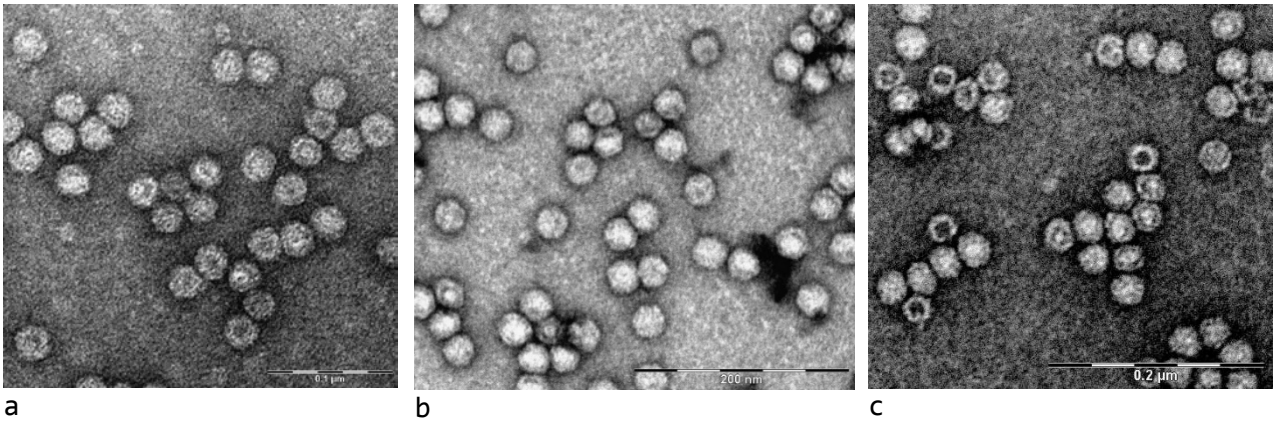
To characterize the antigenic structure of NLP-forming HPIV2 N protein, a panel of monoclonal antibodies was generated after immunizing the mice with the recombinant antigen. The majority of the monoclonal antibodies raised against the recombinant NLPs recognized HPIV2-infected cells, suggesting the antigenic similarity between the recombinant and virus-derived HPIV2 N protein. Fine epitope mapping revealed the C-terminal part (aa 386–504) as the main antigenic region of the HPIV2 N protein.

HPIV2 and HPIV4 N proteins were evaluated as antigens in the developing of competitive ELISA for detecting of specific IgG in human sera.

Generation of recombinant capsids of parvoviruses

Parvoviruses are among the smallest and simplest eukaryotic DNA viruses, that infect a wide range of species, both vertebrates and invertebrates. Porcine parvovirus (PPV) is a widespread infectious virus that causes serious reproductive diseases of swine and death of piglets. Human parvovirus 4 (HPARV4) and human bocaviruses (HBoV) 1-4 are newly discovered viruses found around the globe in asymptomatic as well as patients with respiratory (HBoV1) or gastrointestinal (HBoV2-4) symptoms. Recent reports suggest the possible role of these visures in encephalitis. Self-assembled virus-like particles (VLPs) composed of the major parvoviral capsid protein VP2 were generated in yeast *Saccharomyces cerevisiae* [Tamošiūnas et al. 2013; 2014; 2016]. Recombinant VLPs were similar to native parvovirus particles in size and morphology (Fig. 6). A collection of VP2-specific monoclonal antibodies was generated using yeast-synthesised VLPs of PPV, HBoV and HPARV4.

Indirect IgG ELISA based on the recombinant VLPs for detection of PPV-specific antibodies in swine sera was developed and evaluated [Tamošiūnas et al. 2014]. The sensitivity and specificity of the new assay were found to be 93.4 % and 97.4 %, respectively. Recombinant HBoV1-4 VP2 VLPs were employed to develop serological assays to detect virus-specific IgG antibodies in human serum specimens [Tamošiūnas et al. 2016]. Competition ELISA format was used in order to eliminate cross-reactivity between bocavirus species. Results revealed that HBoV1 is more prevalent than other HBoV subtypes and majority of tested patient encounter bocaviral infection in early childhood. Recombinant HPARV4 VLPs were employed in evaluation of virus prevalence in Lithuanian population, revealing higher percentage (9.4%) of seropositive low-risk patients than in other European countries. Therefore, yeast *S. cerevisiae* represents a promising expression system for generating recombinant parvoviral VP2 protein VLPs of diagnostic relevance and high antigenicity.



a b c

Fig. 6. Electron micrograph of recombinant PPV (a), HBoV1 (b) and HPAR4 (c) VP2 VLPs generated in yeast. Scale bars 100 nm (a) or 200 nm (b,c).

Analysis of yeast expression mechanism

Yeast expression mechanism has been investigated by different techniques. Two-dimensional gel electrophoresis (2DE) is one of the most popular methods in proteomics. Research group of Dr. Slibinskas R. employed non-equilibrium pH gradient electrophoresis (NEPHGE)-based 2DE technique for studying stress responses in recombinant protein producing yeast. Comparison of broad range (pH 3–10) gradient-based 2DE methods suggested that NEPHGE-based method is preferable over commonly used immobilized pH gradient (IPG)-based 2DE method for the analysis of basic proteins (Fig. 7), [Slibinskas R. et all, 2013].

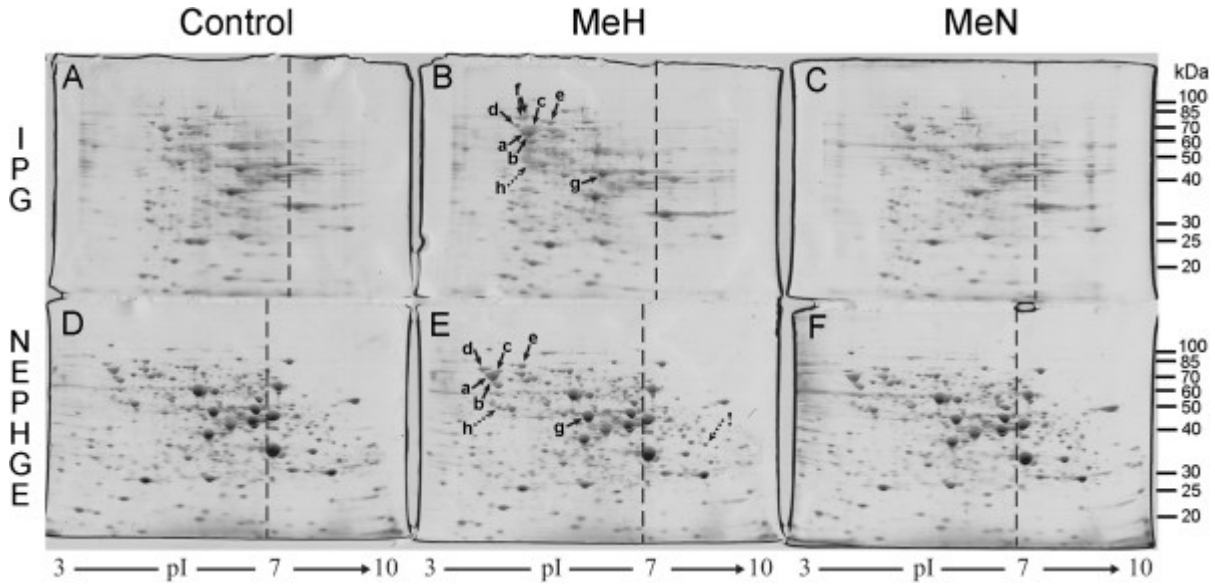


Fig. 7. 2DE of yeast whole cell lysates using IPG (A-C) and NEPHGE (D-F) based methods at high protein load. [*Proteome Sci*. 2013, 11:36].

Plant anthocyanin research

Anthocyanins are essential contributors to the fruit coloration, an important quality feature and a breed determining trait of a sweet cherry fruit. It is well established that the biosynthesis of anthocyanins is regulated by the interplay of specific transcription factors belonging to MYB and bHLH families accompanied by the WD40 protein. In this study, we isolated and analysed WD40, bHLH3, bHLH33 and several closely related MYB10 gene variants from different cultivars of sweet cherry, analysed their expression in fruits with different anthocyanin levels at several developmental stages and determined their capabilities to modulate anthocyanin synthesis in leaves of two Nicotiana species. Our results indicate that transcription level of one MYB10 variant correlates with fruit coloration, but anthocyanin synthesis in Nicotiana was induced by another variant, moderately expressed in fruits. The analysis of two fruit-expressed bHLH proteins revealed that bHLH3 enhances MYB-induced anthocyanin synthesis, whereas bHLH33 has strong inhibitory properties (Fig.8). This study is supported by National Science Program “Healthy and safe food” grant No. SVE-11008.

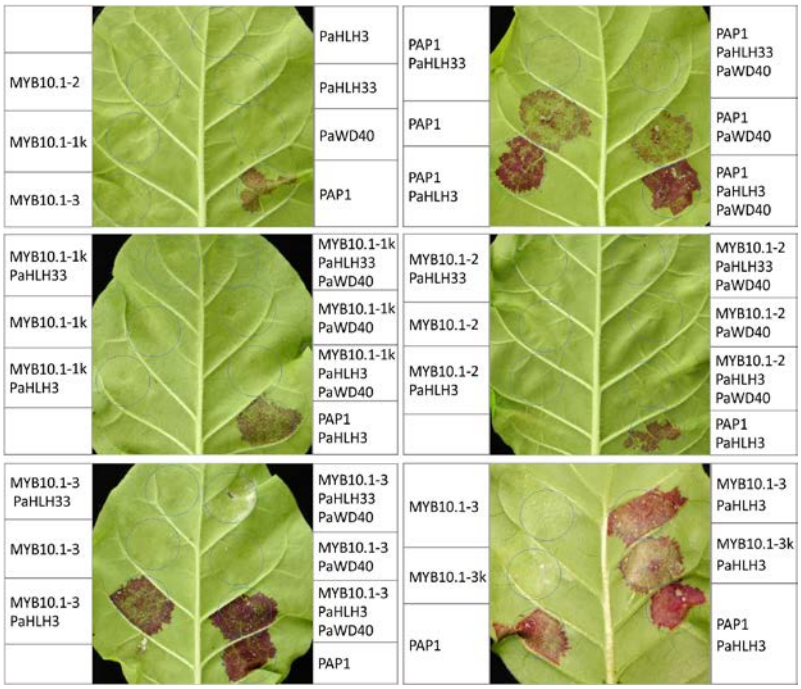


Fig.8. Transient expression of putative regulators of anthocyanin synthesis in leaves of Nicotiana tabacum. Infiltrated control cDNAs: PAP1, Arabidopsis thaliana PAP1 (MYB75); GFP, Aequorea victoria GFP. Tested Prunus avium genes isolated from following cultivars: PaWD40, PaHLH33, MYB10.1-1, MYB10.1-3 from 'Irema BS'; PaHLH3 from 'Kitayanka'; MYB10.1-3 from 'Regina'. MYB10.1-1k – cDNA from 'Kitayanka' fruits; MYB10.1-3k – cDNA expressed in N. benthamiana leaves from infiltrated 'Irema BS' gene

Viroid evolution in the symptomless host

Viroids are small circular RNA able to survive, propagate and cause disease in the susceptible agricultural crops. There were recently reported the multiple plant species acting as the reservoirs for the further viroid spread without any visible symptom expression. We have investigated the possible impact of such reservoir on viroid evolution under the pressure of increasing anthropogenic pollution. Changes in viroid variability caused by chronic, acute and extremely strong oxidative stress induced by an elevated ozone concentration were assessed (Fig.9). Our studies revealed that strong oxidative stress as well as the genotypic peculiarities of host in the symptomless host could significantly alter viroid adaptability and polymorphism.

Fig. 9. Visual injury caused by an acute ozone treatment (400 ppb × 6 h) carried out on grown up plants of tomatoe (*Lycopersicon esculentum* Mill.) cv. Micro-Tom before viroid inoculation.

Studies of plant signaling mechanisms

During plant responses to stress or developmental cues signaling via mitogen-activated protein kinases (MAPKs) mediates fast, precise and specific responses in cells. The mechanism of this signaling in plants is similar as in other eukaryotes and is relatively well understood, whereas termination of this process by the MAPK phosphatases is less known. The study is aimed to understand the biological roles of PP2C-type MAPK phosphatases in the model plant *Arabidopsis thaliana*. We found that PP2C-type phosphatases regulate MAPKs. PP2C can influence cell fate decisions during stomata development. Stomata are cells essential in water/gas exchange between plant and environment and thus supporting our ecosystem. Gene expression analysis enabled identification of specific MAPKs and a PP2C phosphatase induced during stomata cell development [Fuchs S et al., 2013, Schweighofer A et al., 2014]. This study is supported by Lithuanian Science Council grant No. MIP-003/2014. PP2C-type phosphatases also control plant cell signaling pathways in reponse to pathogens. We have identified a novel PP2C function in signaling induced by bacteria. This study is supported by Lithuanian-Swiss Program grant No. CH-3-ŠMM-01/10.

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