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PLANT STRESS UNDER CLIMATE CRISIS

Agricultural proteomic technique for development of flooding-tolerant soybean.

Setsuko Komatsu[1]

[1]Department of Applied Chemistry and Food Science, Fukui University of Technology, Fukui 910-8505, Japan

Email corresponding author: skomatsu@fukui-ut.ac.jp

Climate changes influence the magnitude and frequency of hydrological fluctuations, which create an unfavorable environment for the growth and development of crops. Due to the fact that the accurate analysis of a large number of proteins promotes the knowledge of biological systems, gel-free/label-free proteomics has been performed on agricultural materials. Soybean, which is rich in protein, oil and phytochemicals, is cultivated in several climatic zones. However, its growth is markedly decreased by flooding stress. In the present study, proteomic techniques were used for understanding the flood-response and -tolerant mechanisms in soybean. Under flooding, soybean seedlings showed differential regulation of proteins involved in hormonal signal transduction, transcriptional control, glucose degradation/ sucrose accumulation, alcohol fermentation, gamma-aminobutyric acid shunt, reactive-oxygen species scavenging suppression, mitochondrial impairment, ubiquitin/ proteasome-mediated proteolysis, and cell-wall loosening. Although many flood-response mechanisms have been reported, flood-tolerant mechanisms have not been fully clarified for soybean because of limitations in germplasm with flooding tolerance. Subcellular localizations and post-translational modifications play important role in stress tolerance to flooding stress. Based on the results from proteomic analyses, the roles of key proteins in crops related to stress tolerance were carefully confirmed with molecular biological techniques. These approaches contribute not only to the understanding of stress-tolerant mechanisms in crops, but also to the production of crops with environmental-stress tolerance.

Keywords: agricultural proteomics, soybean, flooding stress, stress-tolerant mechanism.

Deubiquitination of plasma membrane proteins.

Nathalie Berger[1], Vincent Demolombe[1], Valérie Rofidal[1], Sonia Hem[1], Véronique Santoni[1]

[1]IPSiM, Univ. Montpellier, CNRS, INRAE, Institut Agro, Montpellier, France

Email corresponding author: veronique.santoni@inrae.fr

In recent times, the knowledge of the role ubiquitin plays in plant cellular processes has expanded in plants, with one example being in receptor endocytosis and trafficking. In mammals, several deubiquitylating enzymes (DUBs) have been implicated either in facilitating receptor endocytosis and degradation or in rescuing receptor levels by preventing endocytosis and/or promoting recycling to the PM. By contrast, in plants, the role of DUBs, a small family of 57 members, in regulating PM protein abundance and trafficking is by far less known. In order to extend our knowledge of membrane transporters targeted by the DUB UBP13, a K-GG antibody enrichment method integrated with quantitative mass spectrometry (MS) was used to compare the ubiquitylomes of WT and transgenic plants overexpressing UBP13. The quantification of 7000 ubiquitinated peptides belonging to 2350 proteins revealed that 300 proteins could be deubiquitinated by UBP13, mainly enriched in proton pumps, auxin and nutrient transporters among them a nitrate transporter NRT2;1, and trafficking proteins. In addition, the ubiquitination of about 150 PM proteins among them, aquaporin PIP2;1, was enhanced. Combined to a MS quantitative analysis of protein abundance, this analysis allows to propose a dual role for UBP13 in stabilizing or degrading proteins and offers promising insights in the regulatory mechanisms of plant mineral nutrition. A putative regulatory role of UBP13 on PIP2;1 and NRT2;1 function under osmotic stress will be discussed.

Keywords: *Arabidopsis thaliana*, proteomics, ubiquitylome, deubiquitinase, osmotic stress, aquaporin

Acknowledgment: financial support by INRAE, project RUMBA.

Assessment of carbon metabolism of Coffee Kawisari hybrid challenged by Hemileia vastatrix, the causal agent of Coffee Leaf Rust.

Leonor Guerra-Guimarães[1,2], Jéfyne Carrera[1,3], Marina do Rosário Santos[1], Carla Pinheiro[4,5], Manzur -E- Mohsina Ferdous[6], Inês Diniz[1,2], Andreia Loureiro[2], Helena Gil Azinheira[1,2], Thomas Roitsch[6], Maria do Céu Silva[1,2], John D'Auria[7]

[1]Centro de Investigação das Ferrugens do Cafeeiro, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal; [2]Linking Landscape, Environment, Agriculture and Food Research Unit, Associated; Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal; [3]Instituto de Ciências Naturais, Departamento de Biologia, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil; [4]UCIBIO Applied Molecular Biosciences Unit, Department of Life Sciences, NOVA School of Science and Technology, Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal; [5]Associate Laboratory i4HB Institute for Health and Bioeconomy, NOVA School of Science and Technology, Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal; [6]Department of Plant and Environmental Sciences, Section for Crop Sciences, Copenhagen University, Højbakkegård Allé 13, 2630 Taastrup, Denmark; [7]Research Group Metabolic Diversity, Department of Molecular Genetics, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK Gatersleben), OT Gatersleben, Corrensstraße 3, 06466 Seeland, Germany

Email corresponding author: leonorguimaraes@edu.ulisboa.pt

Plants have evolved sophisticated mechanisms to coordinate carbon metabolism during growth and development both under optimal and stress conditions. In coffee-rust biotrophic interactions, plants try to limit pathogen access to nutrients (e.g., sugars and sugar derivatives) and trigger immune responses, while *Hemileia vastatrix* (Hv) attempts to circumvent plant defences and control the host's primary metabolism for its own benefit. Previous proteomics data highlighted the up-regulation of proteins from photosynthesis, primary metabolism, and redox-related enzymes along the coffee resistance response. Coffee Kawisari hybrid - Hv interactions (resistant and susceptible reactions) were evaluated using a single sample fractionation method for metabolite and protein extraction. The microscopic evaluation of the Hv infection process revealed that coffee resistance was associated with early hypersensitive response and accumulation of phenolic-like compounds in host cell walls. GC-TOF-MS untargeted metabolomics allowed the identification of metabolic components, such as sugars, sugar derivatives, amino acids, phenylpropanoids, chlorogenic acids, alkaloids, and fatty acids (using the Golm Metabolome database). The overrepresentation of several caffeoylquinic acids in the resistance reaction may be linked to the accumulation of the phenolic-like compounds that were cytologically observed at the infection sites. Furthermore, sugar-related features also played a role in distinguishing between resistant and susceptible reactions, such as glucose and galactose. The cellular availability of mono and disaccharides is the result of the activity of several enzymes, e.g., sucrose synthase and invertases, that can be targeted by Hv in its strategy to manipulate plant carbon metabolism. The activity profile of these enzymes along the infection will be discussed. Proteomic analysis of the same samples (using the single sample fractionation method previously mentioned) is foreseen. The ultimate goal is to establish a connection between the metabolite and protein signatures.

Keywords: Resistance and susceptible reactions, cytology, semi-high throughput enzyme activity profiling, GC-TOF-MS untargeted metabolomics.

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A proteomic and phenotypic analysis of hypoxia tolerance in *Arabidopsis*.

Hongtao Zhang[1], Chelsea Rundle[1], Briony Parker[1], Rashmi Sasidharan[2], Frederica L. Theodoulou[1]

[1]Plant Sciences and the Bioeconomy, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK
[2]Plant-Environment Signaling, Institute of Environmental Biology, Utrecht University, Utrecht, 3584 CH, The Netherlands

Email corresponding author: Hongtao.zhang@rothamsted.ac.uk

Climate change has increased the frequency and severity of flooding events, with significant negative impacts on global agricultural productivity. Understanding how to improve plants' survival after flooding is crucial for food security. Many plants, including *Arabidopsis thaliana* exhibit natural variation in flooding tolerance. Post-transcriptional regulation is important during flooding-generated hypoxia, and protein dynamics during stress/recovery are incompletely understood. In this study, we established hypoxia conditions and phenotyping assays and tested 19 accessions which are the founders of a Multi-parent Advanced Generation Inter-Cross (MAGIC) population. Hypoxia tolerance was largely consistent with submergence tolerance among the previously tested accessions. Quantitative proteomics was performed to study protein dynamics in one sensitive (Ler-0) and two tolerant founders (Col-0 and Zu-0) in response to hypoxia. This has established the basis for a hypoxia screen and QTL analysis of the MAGIC population in future work. The longer-term goals of the project are to identify mechanisms underpinning hypoxia tolerance and to inform breeding of flood tolerant crops.

Keywords: *Arabidopsis Thaliana*, MAGIC, hypoxia, proteomics, phenotyping.

Acknowledgment: Work supported by the Rothamsted Institutional Sponsorship Fund (ISF) RP10667-25.

Multi-omics combined with genetic analysis reveals key transcription factors involved in the acclimation of olive plants to cold stress.

Christina Skodra[1], Michail Michailidis[1], Adrian Mehmeti[2], Martina Samiotaki[3], Ioannis Ganopoulos[4,5], Georgia Tanou[5,6], Christos Bazakos[4,5,7], Sotirios Fragkostefanakis[2], Athanassios Molassiotis[1]

[1]Laboratory of Pomology, Department of Horticutlure, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece
[2]Plant Molecular and Cell Biology, Goethe University, Frankfurt am Main 60438, Germany
[3]Institute for Bioinnovation, Biomedical Sciences Research Center "Alexander Fleming", Vari 16672, Greece
[4]Institute of Plant Breeding and Genetic Resources, ELGO-DIMITRA, Thessaloniki-Thermi, 570001, Greece
[5]Joint Laboratory of Horticulture, ELGO-DIMITRA, Thessaloniki-Thermi 57001, Greece
[6]Institute of Soil and Water Resources, ELGO-DIMITRA, Thessaloniki-Thermi, 57001, Greece
[7]Max Planck Institute for Plant Breeding Research, Department of Comparative Development and Genetics, Cologne 50829, Germany

Email corresponding author: amolasio@agro.auth.gr

Low temperature is a major factor that influences the development of olive (*Olea europaea* L.) trees during cold seasons. However, the way olive plants regulate homeostasis during cold acclimation remains unclear. In the current study, we investigated the responses of olive (cv. "Chondrolia Chalkidikis") plants to cold exposure. One-year-old olive trees were transferred to a growth chamber and experienced a gradual temperature decrease, from 15 to -3 ºC, for 35 days. In parallel, another group of trees was kept under the same light/humidity conditions at 20 ºC (control). The physiological status of both groups of plants (cold-stressed and non-stressed) was evaluated using physiological indexes. Wide transcriptomic and proteomic analyses were performed in cold stressed leaves exposed to 5, 0 and -3 ºC. Combined analysis of the generated -omics data unraveled molecular pathways that were activated during cold acclimation. Integrating multiple types of -omic data together, using biological knowledge and computational models, allowed us to identify key transcription factors (TFs) involved in the acclimation of the olive trees to low temperatures. Among them, two ethylene response transcription factors, namely ERF1 and ERF5, were remarkably differentiated and were further functionally analyzed to investigate their regulatory role during cold acclimation. Using functional characterization approaches, we found that tomato protoplasts overexpressing either ERF1 or ERF5 increased their viability during cold exposure. Promoter activation assays of *in silico* predicted ERF1/ERF5-targets uncovered cold-responsive genes, providing a refined blueprint of the molecular acclimation of the olive tree during cold.

Keywords: Abiotic stress, ethylene response transcription factors, *Olea europaea* L., omics analysis.

Acknowledgments: This work was supported by the Hellenic Foundation for Research and Innovation (HFRI) under the HFRI PhD Fellowship grant (Fellowship Number: 10687).

Characterization of The Enzymes in Monolignol and Flavonoid Biosynthetic Pathways in Sorghum.

ChulHee Kang[1], Scott E. Sattler[2], Wilfred Vermerris[3]

[1]Department of Chemistry, Washington State University, Pullman WA 99164
[2]U.S. department of Agriculture – Agricultural Research Service, Wheat, Sorghum, and Forage Research Unit Lincoln, NE 68583
[3]Department of Microbiology & Cell Science, UF Genetics Institute and Florida Center for Renewable Chemicals and Fuels, University of Florida, Gainesville, FL 32610

E-mail corresponding author: chkang@wsu.edu

Sorghum is an attractive feedstock for bioenergy and flavonoid production due to its high biomass yield, drought tolerance, and efficient use of soil nitrogen. Especially when grown on low-productivity land, cultivation of sorghum can ease the overall environmental impact and adverse effects on food supplies. However, the efficiency of current biomass conversion methods is hindered by the presence of lignin. Decreasing lignin content and altering lignin subunit composition can significantly benefit conversion efficiency. In addition, flavonoids are potent antioxidants, which play a role in defense against pathogens and insects, attracting pollinators, protection against UV-radiation and reactive oxygen species. We rationally modified monolignol and flavonoid pathway enzymes based on detailed structural knowledge in order to balance biomass conversion efficiency and plant fitness. We focused on a fundamental understanding of several key enzymes: PAL, C4H, C3H, CCoAOMT, CCR, COMT, CAD, APX and PRX in lignin biosynthesis; CHS, CHI, F3H, DFR, FLS, ANS, ANR, FNR, F3′5′H, F3′H and FNSII in flavonoid biosynthesis. Elucidation of the catalytic mechanisms of these enzymes will enable the identification of the most suitable targets for protein engineering. The ultimate goal is to improve biomass conversion efficiency that can be used as a source for renewable fuels, chemicals and animal feedstock, and the development of modified enzymes that can be used for the production of (novel) compounds with economic value, without compromising crop yield. Taken together, this approach identified critical residues and explained substrate preferences among isozymes in sorghum and other monocots.

Keywords: *Soghum bicolor,* lignin, monolignol/flavonoid biosynthetic pathway.

Acknowledgment: Work supported by the NSF and USDA.

Flowering Under Stress: phenomics and proteomics approaches to chickpea grain yield and quality.

Carla Pinheiro[1,2], Isabel Duarte[3], Leonor Guerra-Guimarães[4], Isa C. Ribeiro[5], Mª Graça Carvalho[6], Sébastien Planchon[7], Jenny Renaut[7], Thomas Roitsch[8]

[1]UCIBIO - Applied Molecular Biosciences Unit, Department of Life Sciences, NOVA School of Science and Technology, Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal; [2]Associate Laboratory i4HB - Institute for Health and Bioeconomy, NOVA School of Science and Technology, Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal; [3]INIAV - Instituto Nacional de Investigação Agrária e Veterinária, Elvas, Apartado 6, 7350-951 Elvas, Portugal; [4]LEAF - Linking Landscape, Environment, Agriculture and Food & Associated Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal; [5]Instituto de Tecnologia Química e Biológica, Universidade NOVA de Lisboa, Av. da República, EAN, 2781-901 Oeiras, Portugal; [6]Instituto Politécnico de Portalegre, 7300-110, Portalegre, Portugal; [7]Luxembourg Institute of Science and Technology, 5, Avenue des Hauts-Forneaux, L-4362 Esch/Alzette, Luxembourg; [8]Department of Plant and Environmental Sciences, Copenhagen University, Højbakkegård Allé 13, 2630 Tåstrup, Denmark

Email corresponding author: cm.pinheiro@fct.unl.pt

Chickpea (*Cicer arietinum*) is a major player in the FAO Zero Hunger program "toolkit". It is the second most cultivated food legume, a source of sustainable protein (and other nutrients) and contributes to improved soil health and lower fertilization input. Grain yield, seed protein content and nutritional quality were found to be largely affected by Genotype x Environment. In the Mediterranean regions, higher frequency, intensity and duration of droughts, as well as hotter droughts are being registered, thus interfering with agroecosystems' structure, composition, and functions. A controlled conditions assay (non-invasive phenotyping@PhenoLab) was undertaken in two genotypes from the Portuguese chickpea breeding program (Elvas, INIAV). Results revealed that high temperature (32º C during the reproductive phase) and water regime (40% vs 10% soil water content) play a significant role in chickpea development, seed production and composition, and protein content. As seed biochemical signatures allow to discriminate between genotypes, the power protein-based methodologies in genotype assessment are highlighted. Because some proteins can resist gastrointestinal digestion and influence human health, the processed seed proteome (i.e. following soaking, boiling and *in-vitro* digestion) in three consecutive years was further analysed in field-grown chickpeas (Elvas, INIAV). The seed proteome was found to be highly conserved, with minor changes being attributed to the seed development conditions. Furthermore, *in vitro* digestion efficiently removed many anti-nutritional proteins. Combined phenotyping and omic´s approaches contribute to the mechanistic knowledge of how severe terminal drought and high temperature modulate sink capacity and productivity (yield and quality). The integrated use of phenomics and omics methodologies has significant potential to increase our understanding of plant growth and development and, thus, an efficient, knowledge-based management of crops and resources.

Keywords: phenotyping, proteomics, breeding programs.

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The cAMP-dependent phosphorylation footprint in the response to heat of tobacco cells.

Guido Domingo[1], Milena Marsoni[1], Maria Concetta De Pinto[2], Stefania Fortunato[2], Marcella Bracale[1], Candida Vannini[1]

[1]Department of Biotechnology and Life Science, University of Insubria, Varese, Italy
[2]Department of Biology, University of Bari "Aldo Moro", Bari, Italy

Email corresponding author: g.domingo@uninsubria.it

The 3′,5′-cyclic adenosine monophosphate (cAMP) is increasingly recognized as an essential signaling molecule in plants, where cAMP-dependent processes also include responses to environmental stimuli. Nevertheless, many components of this signal transduction are still a mystery. In this study, tobacco Bright Yellow-2 cells overexpressing a genetic tool that buffers the intracellular levels of cAMP, have been used to explore the phosphorylation changes induced by cAMP in control conditions or following heat stress. Our data showed that the plant kinome is perturbed by cAMP depletion and kinases, such as mitogen-activated protein kinases, receptor-like kinases and calcium-dependent protein kinases, play active roles in the signaling process. The *in vivo* cAMP dampening affected the mRNA processing through the modulation of the phosphorylation status of several RNA-binding proteins involved in splicing, including many SR proteins. Moreover, our results showed a significant overlap between cAMP- and temperature-responsive phosphosites; however, several proteins are phosphoregulated in the opposite way by cAMP depletion and heat stress. Finally, a two-way ANOVA analysis allowed us to distinguish phosphoproteomic changes that represent the specific impact of cAMP depletion on the heat stress response. Overall, our results provide new insights into the molecular mechanism by which cAMP affects the heat stress response in plants.

Keywords: cyclic AMP, phosphoproteomics, heat stress, signaling, tobacco BY-2 cells.

Towards characterizing the molecular mechanism of cold-stress induced scald in apples.

Christina Skodra[1], Evangelos Karagiannis[1,2], Michail Michailidis[1], Martina Samiotaki[3], Ioannis Ganopoulos[4,5], Georgia Tanou[5,6], Christos Bazakos[4,5,7], Athanasios Dalakouras[8], Athanassios Molassiotis[1]

[1]Laboratory of Pomology, Department of Horticutlure, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece
[2]Department of Agriculture, University of Western Macedonia, Florina 53100, Greece
[3]Institute for Bioinnovation, Biomedical Sciences Research Center "Alexander Fleming", Vari 16672, Greece
[4]Institute of Plant Breeding and Genetic Resources, ELGO-DIMITRA, Thessaloniki-Thermi, 570001, Greece
[5]Joint Laboratory of Horticulture, ELGO-DIMITRA, Thessaloniki-Thermi 57001, Greece
[6]Institute of Soil and Water Resources, ELGO-DIMITRA, Thessaloniki-Thermi, 57001, Greece
[7]Max Planck Institute for Plant Breeding Research, Department of Comparative Development and Genetics, Cologne 50829, Germany
[8] Institute of Industrial and Forage Crops, ELGO-DIMITRA, Theofrastou Str., 41335, Larisa, Greece

Email corresponding author: amolasio@agro.auth.gr

Superficial scald is a major physiological disorder of apple fruit (*Malus domestica Borkh.*) characterized by skin browning after prolonged cold storage; however, the cascade of the molecular processes that modulate scald development remains unclear. To gain insight into the mechanisms underlying scald development, 'Granny Smith' apples were harvested at two maturity stages (early and late harvest), then cold stored (0 ºC for 3 months) and subsequently ripened at room temperature (20 ºC). Phenotypic and physiological data indicated that early harvest apples inhibited a decreased ethylene-dependent ripening behavior and an increase in scald development. A wide combination of multi-omic analysis (transcriptional, epigenetic, proteomic and metabolic) in apple skin tissue enabled characterization of potential genes, proteins and metabolites that were altered in scald (early harvest) and non-scald (late harvest) phenotypes at pro-symptomatic (during cold storage) and scald-symptomatic (ripening) period. Through this approach, we revealed novel scald-associated genes, proteins and metabolic pathways. Also, *in silico* evidence is presented supporting that oxidation-based protein post-translation modifications are involved in scald development. In addition, we functionally characterized through RNA interference (RNAi) approach during various post-harvest stages the active role of *MdAFS* and *MdPPO* in scald expression signatures. The combination of large scale -omics and functional characterization approaches may contribute to understanding the molecular mechanism of superficial scald development in apple fruit.

Keywords: Superficial scald, Granny Smith, epigenetics, transcriptomics, proteomics, RNA interference, functional characterization.

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Integrative transcriptomic and TMT-based proteomic analysis reveals the desiccation tolerance in *Ramonda serbica* Panc.

Marija Vidović[1], Ilaria Battisti[2,3], Ana Pantelić[1], Filis Morina[4], Giorgio Arrigoni[2,3], Antonio Masi[5], Sonja Veljović Jovanović[6]

[1]Institute of Molecular Genetics and Genetic Engineering, Laboratory for Plant Molecular Biology, University of Belgrade, Vojvode Stepe 444a, 11042, Belgrade, Serbia
[2]Department of Biomedical Sciences, University of Padova, Via Ugo Bassi, 58/B 35131 Padova, Italy
[3]Proteomics Centre, University of Padova and Azienda Ospedaliera di Padova, via G. Orus 2/B, 35129 Padova, Italy
[4]Biology Center of the Czech Academy of Sciences, Institute of Plant Molecular Biology, Department of Plant Biophysics and Biochemistry, Branišovská 31/1160, 370 05 České Budějovice, Czech Republic
[5]Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova, Viale dell'Università 16, Legnaro, Padova, Italy
[6]Institute for Multidisciplinary Research, Department of Life Science, University of Belgrade, Kneza Višeslava 1, 11000 Belgrade, Serbia

Email corresponding author: mvidovic@imgge.bg.ac.rs

Ramonda serbica Panc. is a resurrection plant that can survive long periods of desiccation and fully restores its metabolic functions just one day after watering. The aim of this study was to identify key candidates and metabolic pathways involved in *R. serbica* desiccation tolerance. We combined differential transcriptomics and proteomics with the analysis of phenolics, sugars, cell wall polymers and photosynthetic electron transport (PET) chain. TMT-based proteomic analysis allowed the relative quantification of 1192 different protein groups, 408 of which were differentially abundant between hydrated (HL) and desiccated leaves (DL). Almost all differentially abundant proteins and transcripts related to photosynthetic processes were downregulated in DL. Chlorophyll fluorescence measurements showed a shift from linear PET to cyclic electron transport (CET). The levels of H_2O_2-scavenging enzymes, ascorbate-glutathione cycle components, catalases, peroxiredoxins, Fe-, and Mn superoxide dismutase (SOD) were reduced in DL. However, six germin-like proteins (GLPs), four Cu/ZnSOD isoforms, three polyphenol oxidases, and 22 late embryogenesis abundant proteins (LEAPs; mainly LEA4 and dehydrins), were desiccation-inducible. Desiccation led to cell wall remodelling related to GLP-derived H_2O_2/$HO^{\bullet}$ activity and pectin demethylesterification. This comprehensive study contributes to understanding the role and regulation of important metabolic pathways during desiccation with the final aim to help improving the drought tolerance in crops.

Keywords: cell wall remodelling, drought, germin-like proteins, late embryogenesis abundant proteins, resurrection plant.

Acknowledgement: This work was supported by the Science Fund of the Republic of Serbia (PROMIS project LEAPSyn-SCI, grant number 6039663) and by the Ministry of Education, Science and Technological Development, the Republic of Serbia (Contract No. 451-03-68/2022-14/200042, 2022). M.V. wishes to acknowledge the support of COST Action BM1405 for approving STSM in Padua during 2017 and 2018.

How do plants feel the heat and survive? Detection of plant epimutants reversibly defective in the heat-shock response.

Anthony Guihur[1], Baptiste Bourgine[1], Pierre Goloubinoff[1]

[1]Department of Plant Molecular Biology, Faculty of Biology and Medicine, University of Lausanne, CH-1015 Lausanne, Switzerland.

Email corresponding author: Pierre.Goloubinoff@unil.ch

Global warming urges for a better understanding of the molecular mechanisms by which higher plants sense and respond to sharp increments in the ambient temperature and appropriately accumulate protective heat-shock proteins (HSPs) and metabolites. We designed a transgenic *Arabidopsis thaliana* reporter line expressing from a conditional heat-inducible promoter, a fusion gene encoding for nano-luciferase and D-amino acid oxidase, whose expression in the presence of D-valine is toxic. Whereas at 22°C, seedlings grew unaffected by D-valine and did not emit light, most seedlings survived iterative heat shocks without D-valine and strongly emitted light. Unexpectedly as many as 2% seedlings survived heat shocks on D-valine. Yet, in presence of a DNA-methylation inhibitor, seedling survival to heat shocks on D-valine was 0.01%. RNAseq analysis showed that heat-shock survivors on D-valine did not undergo a Heat Shock Response (HSR) and failed to over-express hundreds of HSP genes. Yet, whereas some epi-mutants reverted and could express again a full HSR at the rosette stage, others kept repressing the HSR during their entire life and transmitted their HSR inhibition phenotype to up to 50% of their first-generation descendants. Our results suggest that higher plants can activate a general epigenetic program that can block the entire HSR and transmit this epigenetic phenotype to the next generation. The results suggest that excessive accumulation of thermo-protective HSPs, molecular chaperones in particular, during iterative heat stresses has a strong cost on plant fitness.

Keywords: *Arabidopsis thaliana*, Heat-shock sensing, and signaling, expressomics, epigenetic methylation.

Acknowledgment: Work supported by the Swiss national Fund (31003A_175453).

Post-translational regulation of PDR9/ABCG37, a key player in plant Fe nutrition.

Meijie Li[1], Kevin Robe[1], Shunsuke Watanabe[1], Nathalie Berger[1,2], Vincent Demolombe-Liozu[1,2], Sonia Hem[1,2], Valérie Rofidal[1,2], Esther Izquierdo[1], Véronique Santoni[1,2], Christian Dubos[1,2]

[1]IPSiM, Univ. Montpellier, CNRS, INRAE, Institut Agro, Montpellier, France
[2]MSPP, Univ. Montpellier, CNRS, INRAE, Institut Agro, Montpellier, France

Email corresponding author: Christian.dubos@inrae.fr

Fe is a micronutrient that is essential for crop productivity and the quality of their derived products. This is because Fe is a cofactor for several metalloproteins involved in diverse physiological processes (e.g. photosynthesis, respiration, nitrogen assimilation, etc.). Despite its high abundance in Earth's crust, Fe is poorly available to plants because at neutral to alkaline pH Fe is mostly present in the form of (hydr)oxides that are not readily available for plants. This is the case in calcareous soils that represent one-third of the world's cultivated lands. As a consequence, plants growing on these soils experience Fe deficiency affecting their growth and development. To cope with this poor bioavailability, non-grass species have evolved a reduction-based mechanism to mine Fe from the soil. Nevertheless, the efficiency of this mechanism is optimal at acidic pH. We have recently demonstrated that at alkaline pH, Fe nutrition of *Arabidopsis thaliana* plants relies on the secretion into the rhizosphere of Fe-mobilizing coumarins (secondary metabolites derived from the phenylpropanoid pathway) via the PDR9/ABCG37 transporter. How the activity of this transporter is regulated is still a matter of debate. By using different immunoprecipitation approaches we have identified post-translational modifications (PTMs, phosphorylations and ubiquitinations) on PDR9/ABCG37. Mutated versions of PDR9/ABCG37 at these PTM sites fused with the GFP reporter protein highlighted that the polar localization of PDR9/ABCG37 at the root cortex and epidermis cells was conserved. However, the mutated versions of PDR9/ABCG37 failed to complement *pdr9* loss-of-function mutant. Taken together, these data indicate that the identified PTMs most probably play a key role in regulating the activity of PDR9/ABCG37, and thus plant Fe nutrition.

Keywords: *Arabidopsis thaliana*, proteomics, iron, nutrition, coumarin, PDR9, ABCG37.

Proteomics Evidence of a Systemic Response to Desiccation in the Resurrection Plant *Haberlea rhodopensis*.

Petko Mladenov[1,2,3,5], Norbert Rolland[3], Jenny Renaut[4], Ryozo Imai[5], Dimitar Djilianov[1], Xin Deng[2]

[1]Agrobioinstitute, Agricultural Academy Bulgaria, 1164 Sofia, Bulgaria
[2]Key Laboratory of Plant Resources, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China
[3]Laboratoire de Physiologie Cellulaire et Végétale, University Grenoble Alpes, CNRS, INRAE, CEA, 38054 Grenoble, France
[4]Environmental Research and Innovation (ERIN) Department, Luxembourg Institute of Science and Technology, L-4362 Esch-sur-Alzette, Luxembourg
[5]NARO Institute of Agrobiological Sciences (NIAS), Tsukuba, 305-0086, Japan

Email corresponding author: mladenovpetko@yahoo.com

Global warming and drought stress are expected to have a negative impact on agricultural productivity. Desiccation-tolerant species, which are able to tolerate the almost complete desiccation of their vegetative tissues, are appropriate models to study extreme drought tolerance and identify novel approaches to improve the resistance of crops to drought stress. According to the systems biology approach, genomics tells what can happen, transcriptomics what appears to be happening, metabolomics what has happened, and proteomics what makes it happen. To better understand what makes resurrection plants extremely tolerant to drought, we combined proteomics studies including organellar and phosphorylation proteomics with previously published transcriptomic and metabolomics data from the resurrection plant *Haberlea rhodopensis*. The results revealed new evidence about organelle and cell preservation, posttranscriptional and posttranslational regulation, photosynthesis, primary metabolism, phagocytosis, and cell death in *H. rhodopensis*. These findings provide multi-omics evidence about targets for further genomic and evolutionary studies among resurrection plants and targeted genome editing to improve crop drought tolerance.

Keywords: Resurrection plants, Proteomics, Systems Biology, Drought.

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Impact of Rhamnolipids (RLs), Natural Defense Elicitors, on Shoot and Root Proteomes of *Brassica napus* by a Tandem Mass Tags (TMTs) Labeling approach.

Elise Pierre[1,2,3,†], Paulo Marcelo[2,†], Antoine Croutte[2], Morgane Dauvé[3], Sophie Bouton[1], Sonia Rippa[3], Karine Pageau[1]

[1]Unite Transfrontaliere BioEcoAgro, BIOlogie des Plantes et Innovation (BIOPI), UMRt 1158, Universite de Picardie Jules Verne, 80039 Amiens, France
[2]Plateforme d'Ingenierie Cellulaire & Analyses des Proteines ICAP, FR CNRS 3085 ICP, Universite de Picardie Jules Verne, 80039 Amiens, France
[3]Unite de Genie Enzymatique et Cellulaire, UMR CNRS 7025, Alliance Sorbonne Universites, Universite de technologie de Compiegne, 60203 Compiegne, France

Email corresponding authors: sonia.rippa@utc.fr, karine.pageau@u-picardie.fr

The rapeseed crop is susceptible to many pathogens such as parasitic plants or fungi attacking aerial or root parts. Conventional plant protection products, used intensively in agriculture, have a negative impact on the environment as well as on human health. There is therefore a growing demand for the development of more planet-friendly alternative protection methods such as biocontrol compounds. Natural rhamnolipids (RLs) can be used as elicitors of plant defense mechanisms. These glycolipids, from bacterial secretome, are biodegradable, non-toxic and are known for their stimulating and protective effects, in particular on rapeseed against filamentous fungi. Characterizing the organ responsiveness to defense-stimulating compounds such as RLs is missing. This analysis is crucial in the frame of optimizing the effectiveness of RLs against various diseases. A Tandem Mass Tags (TMT) labeling of the proteins extracted from the shoots and roots of rapeseed has been performed and showed a differential pattern of protein abundance between them. Quantitative proteomic analysis highlighted the differential accumulation of parietal and cytoplasmic defense or stress proteins in response to RL treatments with a clear effect of the type of application (foliar spraying or root absorption). These results must be considered for further use of RLs to fight specific rapeseed pathogens.

Keywords: *Solanum melongena*, proteomics, MAGIC, core collection, GWAS.

Acknowledgment: Work supported by the SHIELD Project ANR Ecophyto-Maturation (ANR-21-ECOM-0006), co-funded by the OFB (Office français de la biodiversité) via the fee for diffuse pollution under the French plan Ecophyto. This study was supported by equipment co-founded by the Regional Council of Picardy and European Union within the CPER 2007–2020.

PLANT INTERACTIONS AND SIGNALING

Unravelling temperature-mediated signalling in plants.

Ive De Smet[1,2]

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, B-9052 Ghent, Belgium
[2]VIB Center for Plant Systems Biology, B-9052 Ghent, Belgium

Email corresponding author: ive.desmet@psb.vib-ugent.be

Plants are exposed to environmental stresses, such as low or high temperature and drought, and continuously respond to changing conditions to prevent damage and maintain optimal performance. To identify regulators of temperature-mediated signalling that are conserved in flowering plants, we mapped changes in protein phosphorylation in both dicots and monocots exposed to warm temperature. The identification of conserved regulators expands our knowledge of temperature-mediated signalling beyond the well-studied pathways and contributes to ensuring food security under a changing climate. Here, I will present our latest work on a signaling axis that is involved in resolving the conflict when drought and high temperature are simultaneously imposed on stomatal aperture control. In addition, I will elaborate on computational approaches to infer signaling networks based on time-resolved phosphoproteome data.

Keywords: *Arabidopsis thaliana,* wheat, soybean, phosphoproteomics, kinase.

Molecular analysis of *B. cinerea* bioactive peptides and its role during infective process.

Almudena Escobar[1], Andrea de Mateo Sanchez[1], Francisco Javier Navas López[1], Mary Cuiñas Diaz[1], Francisco Javier Fernández Acero[1]

[1]Microbiology and Proteomic Laboratory, Institute for Viticulture and Agri-Food Research (IVAGRO), Department of Biomedicine, Biotechnology and Public Health, University of Cádiz, 11510 Puerto Real, Spain

Email corresponding author: franciscojavier.fernandez@gm.uca.es

Bioactive peptides are sequences of amino acids that are normally buried in the structure of parent proteins. They are released as the result of the claveage of proteins sources by proteases from different microorganisms. As a result of this hydrolysis, the peptides become active, showing important roles as agonist and antagonist of opioids, antioxidants, anticoagulants, regulators of cholesterol, and blood pressure, or as antifungals and antibiotics. Another group of peptides is "de novo" produced in microorganism by peptide synthetases. These are called nonribosomal (NRPS) peptides, presenting also a wide range of functions. The potential use of *B. cinerea* to produce "bioactive peptides" has been previously reported. However, its presence and role during the infective cycle is still unclear. This work proposes the isolation and characterization of bioactive peptides from *B. cinerea* cultures addition with or without exogenous protein sources. For bioactive peptide production, *B. cinerea* were cultured in the presence of tomato fruits (as a source of vegetable proteins) and glucose as control. Filtrated supernatant was loaded in centrifugal device filters (cut-off 3kDa) to the recovery of peptides, removing intact proteins. This peptide mixture was cleaned by acetone precipitation. Obtained peptides were used in tomato phytotoxic assays and antibiograms. Those experiments showed that *B. cinerea* is able to produce peptides with phytotoxic and antibiotic activities, either by degrading plant proteins (TCW) or by "de novo" synthesis (GLU). Then, peptide extracts were analyzed by LC-MS/MS. In addition, transcriptional analysis of NRPS genes annotated in the *B. cinerea* genome under both culture conditions was performed to complement the results of the proteomic assay. This approach will represent, for the first time, the role of these peptides during the infection cycle; and highlight the possible biotechnological application of this process in biomass valorization through the transformation of industrial plant residues into bioactive compounds.

Keywords: *Botrytis cinerea*, bioactive peptides, applications, application of peptides, infection tool.

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Defining remodeling of the global temporal proteome responses of Fusarium head blight-resistant and -susceptible wheat cultivars.

Boyan Liu, Reid Buchanan, Payton Curtis, Rebecca Shapiro, Mitra Serajazari, Jennifer Geddes-McAlister

University of Guelph

Email corresponding author: bliu13@uoguelph.ca

The fungal pathogen, *Fusarium graminearum*, is the primary causative agent of Fusarium head blight (FHB) in cereal crops around the world. Infection with the host poses a threat to food security by reducing the crop yield and contaminating food products with mycotoxins, such as deoxynivalenol (DON). Globally, the destruction caused by FHB is expected to increase in severity in the coming years due to a connection with climate change, which requires the need for innovative strategies to combat the disease. Our research uses state-of-the-art mass spectrometry-based proteomics combined with tandem mass tag labeling to define the temporal and resistance-specific host-pathogen interactions between *F. graminearum* and *Triticum aestivum* (wheat). We profiled core proteome defense responses independent of cultivar but regulated across time (24 vs. 120h post inoculation), along with cultivar-specific responses to infection. We found up-regulated proteins in the resistant cultivar that contributes to the early pathogen detection (e.g. Leucine-rich repeat domain containing protein) at 24 hpi and putative defense response proteins (Putative defensin) at 120 hpi. On the pathogen side, comparison of *in vitro* fungal proteome vs. infectome revealed uncharacterized fungal proteins involved in the disease progression. Presently, we are constructing mutant fungal strains using CRISPR-Cas9 and assessing defects in virulence. Overall, results from this study aim to enhance effectiveness of cultivar resistance, reduce severity of infection, and develop strategies to prevent fungal disease in cereal crops.

Keywords: Fusarium head blight, *Fusarium graminearum*, *Triticum aestivum*, Host-pathogen interaction.

First description of *Botrytis cinerea* extracellular vesicles proteome and its role in the infection process.

Almudena Escobar[1], Anne Harzen[2], Sara Christina Stolze[2], Hirofumi Nakagami[2], Francisco Javier Fernández Acero[1]

[1]Microbiology and Proteomic Laboratory, Institute for Viticulture and Agri-Food Research (IVAGRO), Department of Biomedicine, Biotechnology and Public Health, University of Cádiz, 11510 Puerto Real, Spain. [2]Proteomics Group, Max Planck Institute for Plant Breeding Research, Cologne, Germany

Email corresponding author: almudena.escobar@uca.es

Extracellular vesicles (EVs) are membranous particles released by different organisms. EVs carry proteins, lipids, nucleic acids, and carbohydrates implicated in cell communication. In recent years, EVs have become an important topic in the study of pathogenic fungi, due to their relation with the fungal-host interactions. One of the essential research areas in this field is the characterization of the protein profile of EV cargo, because plant fungal pathogens rely heavily on secreted proteins to invade their hosts. However, little is known about EVs of Botrytis cinerea, which is one of the most devastating phytopathogenic fungi. So, the present study has two objectives: (i) the first description of B. cinerea extracellular vesicles; and (ii) the description of their potential role in its infective process. To this aim, B. cinerea was grown in minimal salt medium (MSM) supplemented with 1% of different plant-based elicitors: (i) glucose as a constitutive stage; and (ii) deproteinized tomato cell walls (TCW) as virulence inductor. The isolation of EV was performed by differential centrifugation, filtration, ultrafiltration, and sucrose cushion ultracentrifugation of B. cinerea culture supernatant. Then, EV fractions were visualised by TEM and their cargo was addressed by LC/MS. The used methodology allowed the correct isolation of EVs, with a high number of identified proteins and potential EV markers. The isolated EVs displayed differences in the morphology and protein profile under both assayed conditions. GO analysis showed an enrichment in cell wall metabolism and proteolysis in EV under TCW that was shared with secreted proteins. In addition, KEGG analysis also highlighted the presence of potential virulence/pathogenic factors implicated in cell wall metabolism, among others, in EVs under TCW. In conclusion, this work has revealed that EVs play an essential role in plant-pathogen interaction, working together with the conventionally secreted proteins in crucial steps of the infective process.

Keywords: *Botrytis cinerea*, Extracellular vesicles, proteome, infection tool, cell wall degrading enzyme, unconventional secretion.

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Proteome profiling of arbusculated cells for the in vivo identification of arbuscular mycorrhizal effectors.

Naomi Stuer[1,2], Judith Van Dingenen[1,2], Petra Van Damme[3], Sofie Goormachtig[1,2]

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 71, 9052, Ghent, Belgium
[2]Center for Plant Systems Biology, VIB, Ghent University, Technologiepark 71, 9052, Ghent, Belgium
[3]Laboratory of Microbiology, Department of Biochemistry and Microbiology, Ghent University, 9000 Ghent, Belgium

Email corresponding author: Sofie.Goormachtig@psb.ugent.be

Agricultural application of arbuscular mycorrhizal fungi (AMF) can increase crop productivity through enhancing nutrient and water acquisition without compromising the integrity of the world's ecosystems. Nevertheless, usage of AMF in the field remains limited due to variable crop yield enhancement and difficulty of fungal spore production on a large scale, suggesting a previously unanticipated species specificity during AMF symbiosis. Microbial secreted proteins, so-called 'effector proteins', are crucial regulators of pathogenic and/or symbiotic outcome. Intracellular effectors target host macromolecules in various ways, allowing evasion of the host immune response and modulation of host metabolism and physiology in such a way that a suitable niche for fungal growth is established. The *in silico* prediction of AMF and other fungal effectors, which is often biased towards the presence of a conventional N-terminal signal peptide and effector motifs supposedly involved in effector translocation (e.g. RXLR and LxLFLAK) remains subjected to a consistent error rate. This is primarily due to rapid sequence evolution, a proposed role for unconventional, signal peptide-independent secretion, and effector translocation mediated by unknown fungal sequence motifs. This bottleneck urges the development of unbiased proteomics approaches for the direct, holistic characterization of fungal effectomes. Here, we aim at the direct identification of *Rhizophagus irregularis* cytoplasmic and nuclear effectors in tomato root cells containing arbuscules, intricately branched fungal hyphae that function as the front-line for host-AMF cross-talk and nutrient exchange. To this end, proximity labeling (Turbo-ID) and fluorescence assisted nuclei sorting (FANS) targetted to arbusculated cells will be performed in combination with liquid chromatography tandem mass spectrometry (LC-MS/MS). Downstream functional characterization of identified effectors will enable an enhanced understanding of the molecular mechanisms mediating AMF colonization outcome. Finally, these insights will enable the genetic finetuning of the symbiosis towards enhanced agricultural application.

Keywords: arbuscular mycorrhiza, tomato, effector, Turbo-ID, FANS, LC-MS/MS.

A proteomic study of the intermediate breakdown polypeptides of cowpea main storage protein β-vignin accumulated during seed germination.

Giuditta Carlotta Heinzl, Stefano De Benedetti, Giulia Ceravolo, Francesco Castagna, Davide Emide, Chiara Magni, Alessio Scarafoni

Department of Food, Environmental and Nutritional Sciences, University of Milan, via G. Celoria 2, 20133 Milan, Italy.

Email corresponding author: alessio.scarafoni@unimi.it

The proteomic landscape of mature legume seeds is characterized by the presence of seed storage proteins (SPs), which in cowpea represent about 25% of the dry seed weight. β-vignin is the main SP. SPs are traditionally considered the nitrogen reserve that supports seedling growth during the first steps of germination. This exclusive role has been recently ruled out. In the recent past, much research has been concerned with the study of their deposition during seed development. Very little is known, at the proteomic level, of what happens to SPs during germination. Protein cleavage at germination is believed to occur through a regulated mechanism involving the selective breakdown, operated by specific peptidases, before the native conformation is critically altered to allow the complete hydrolysis, determining the formation of polypeptide fragments. New findings indicate that some of these transiently formed intermediate peptides have been shown to possess specific bioactivities, playing a role in plant defense processes. We undertook the present study as part of our research activity aimed to characterize the molecular relationships between structure and function of SPs. The work investigated the accumulation and stability of the proteolytic products of β-vignin transiently accumulated during seed germination, at different timing, despite the evident degradation of its polypeptides. The main result was the molecular characterization of a 27 kDa intermediate breakdown polypeptide, which, to the best of our knowledge, has never been described before. The intermediate polypeptide seems to retain the trimeric conformation of native β-vignin. The molecular characterization of stable breakdown products is useful to approach the investigation of the functional role during germination, such as insecticidal or antifungal actions, other than the mere source of nitrogen for the seedlings.

Keywords: Seed storage proteins, vicilin, 7S-globulins, germination, proteolysis.

SYSTEMS BIOLOGY

Proteomics-based systems approach of maize response to water deficit.

Mélisande Blein-Nicolas[1], Yacine Djabali[1,2,3], Claude Welcker[4], Llorenç Cabrera Bosquet[4], Stéphane Nicolas[1], Alain Charcosset[1], Michel Zivy[1], Renaud Rincent[1], Marie-Laure Martin[2,3,5]

[1]Université Paris-Saclay, INRAE, CNRS, AgroParisTech, GQE-Le Moulon, 91190, Gif-Sur-Yvette, France
[2]Université Paris-Saclay, CNRS, INRAE, Université Evry, Institute of Plant Sciences Paris-Saclay (IPS2), 91190, Gif sur Yvette, France
[3]Université de Paris Cité, Institute of Plant Sciences Paris-Saclay (IPS2), 91190, Gif sur Yvette, France
[4]LEPSE, INRAE, Univ Montpellier, SupAgro, Montpellier, France
[5]Université Paris-Saclay, AgroParisTech, INRAE, UMR MIA Paris-Saclay, 91120, Palaiseau, France

Email corresponding author: melisande.blein-nicolas@inrae.fr

The evolution of maize yields under water deficit conditions is of particular concern in the context of climate change and human population growth. However, the multiplicity and versatility of drought-response mechanisms make the design of new drought-tolerant varieties a complex task that would greatly benefit from a better understanding of the genotype-phenotype relationship. To provide novel insight into this relationship, we integrated high-throughput phenotypic, proteomics, and genomics data acquired on 254 maize hybrids grown under well-watered (WW) and water deficit (WD) conditions by using association genetics and protein co-expression analysis. By first analyzing the data obtained in each watering condition, we showed that water deficit, even mild, induced a deep proteome remodeling which affected the structure of the protein co-expression network. We also highlighted a genetic architecture reprogramming of many proteins abundance that may affect the phenotypic traits. We then focused on the plants' response to water deficit by analyzing the plasticity of the different genotypes at the proteome and phenotype levels. We showed that the genetic architecture of plasticity indices, computed as WD/WW log ratios for protein abundances and WD/WW ratios for phenotypic traits, do not overlap with those obtained for the same proteins and traits in both WW and WD conditions. Work is currently in progress to build molecular networks linking the genome to the phenotype through the proteome based on the data collected in the WW and WD conditions and the plasticity indices.

Keywords: Z*ea mays*, systems biology, GWAS, multi-omics, plasticity, drought.

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A One Health systems approach towards improved mycotoxin degradation in wheat.

Reid Buchanan[1], Boyan Liu[1], Jiaxi Lu[1], Payton Curtis[1], Danisha Johal[1], Rebecca Stevens-Green[1], Samanta Pladwig[1], Mitra Serajazari[2], David Overy[3], Rebecca Shapiro[1], Jennifer Geddes-McAlister[1,*]

[1]Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada
[2]Department of Plant Agriculture, University of Guelph, Guelph, Ontario, N1G 2W1, Canada
[3]Agriculture and Agri-Food Canada, Ottawa Research and Development Centre, 960 Carling Avenue, Ottawa, Ontario, K1A 0C6

Email corresponding author: jgeddesm@uoguelph.ca

Infection by *Fusarium* spp. across the world's most productive and lucrative cereal crops (e.g., corn, wheat, soybean) leads to millions in annual losses in diminished export and domestic sales associated with yield and quality reduction. For Fusarium head blight (FHB), a fungal disease caused primarily by *Fusarium graminearum*, the production of mycotoxins, such as deoxynivalenol (DON), have severe consequences for the livestock and poultry industries through consumption of contaminated feed and water run-off, as well as human health for consumption of food from contaminated grains (3, 4). Our research used state-of-the-art mass spectrometry-based proteomics combined with tandem mass tags to define changes in plant protein production in the presence of low and high concentrations of 15-acetyl DON over a time course of infection (e.g., 24 and 120 h post inoculation) in both susceptible (i.e., Norwell) and resistant (i.e., Sumai#3) wheat varieties. We quantified over 7,000 plant proteins with thousands showing significant changes in protein abundance across the conditions. Advanced bioinformatics analysis identified over 100 plant proteins demonstrating altered production in response to DON, suggesting a signature of plant defense defined by DON. Next, we will integrate our proteomics and metabolomics datasets to prioritize candidates for characterization using *in vitro* DON degradation assays. Long-term, our work aims to develop new wheat lines through selected breeding that are resistant to the accumulation of dangerous mycotoxins, including DON, through enhanced degradation capabilities to improve crop yield and quality for farmers.

Keywords: *Fusarium graminearum*, *Triticum aestivum* (wheat), mycotoxin, deoxynivalenol, quantitative proteomics, systems biology.

Acknowledgment: Work supported by the Ontario Ministry of Agriculture, Food, and Rural Affairs, Grain Farmers of Ontario, SeCan, Canadian Foundation for Innovation, and the One Health Institute at the University of Guelph.

Plant PTM Viewer 2.0 and a novel DIA-MS method to quantify the degree of cysteine oxidation.

Patrick Willems[1,2,3,4], Jingjing Huang[1,2], An Staes[3,4,5], Lieven Sterck[1,2], Frank Van Breusegem[1,2], Kris Gevaert[3,4]

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052, Ghent, Belgium.
[2]VIB Center for Plant Systems Biology, VIB, 9052, Ghent, Belgium.
[3]Department of Biomolecular Medicine, Ghent University, 9052, Ghent, Belgium.
[4]VIB Center for Medical Biotechnology, VIB, 9052, Ghent, Belgium.
[5]VIB Proteomics Core, VIB, 9052, Ghent, Belgium.

Email corresponding author: Patwille.willems@ugent.be

Post-translational modifications (PTMs) alter the physicochemical properties of proteins and can steer their function in diverse physiological processes. Contemporary mass spectrometry studies routinely report hundreds to thousands of different PTMs. Downstream analyses of these require centralized databases integrating PTM sites. To this end, we developed in 2019 The Plant PTM Viewer (https://www.psb.ugent.be/PlantPTMViewer), outlining thousands PTM sites for 19 PTM types derived from more than 100 large-scale MS studies in five plant species. In version 2.0, three extra plant species, approximately 50 MS studies and 10 PTM types were added. Moreover, we identified new PTMs by bioinformatic re-analysis of a higher-quality MS draft of *Arabidopsis thaliana* using open searches, i.e., ultra-tolerant searches that enabled the identification of mass shifts on peptides caused by PTMs. In this manner, we identified glycosylation, hundreds of lysine crotonylation and hydroxyisobutyrylation sites, and a modification by 3-phosphoglyceryl on lysine hitherto neglected in plants. As described in mammals, this metabolic modification targets glycolytic enzymes in the cytosol, but also plastidic isoforms in plants. Additionally, we developed a novel experimental method to quantify the degree of cysteine oxidation in cell cultures and plant leaves. Relying on the plexDIA algorithm developed for single cell proteomics, we quantified heavy and light isotopically labeled cysteine peptides that reveals the absolute reduction-oxidation state of cysteines. This labeled shotgun approach allows simultaneous quantification of protein levels and cysteine oxidation states in physiologically relevant samples.

Keywords: Cysteine, post-translational modification, Plant PTM Viewer, DIA-MS.

Acknowledgment: Work supported by Research Foundation-Flanders (FWO) Postdoctoral fellowship (no. 12T1722N to P.W.).

Foliar proteome modulation in *Arabidopsis* to rhizobacterium *Pseudomonas simiae* root exposure.

Letizia Bernardo[1], Francesca Marzorati[2], Rossana Rossi[1], PierLuigi Mauri[1], Piero Morandini[2], Irene Murgia[2], Dario Di Silvestre[1]

[1]Proteomic and Metabolomic Laboratory, Institute for Biomedical Technologies-National Research Council (ITB-CNR), Segrate (MI), Italy
[2]Department of Environmental Science and Policy, University of Milan, Milan, Italy

Email corresponding authors: letizia.bernardo@itb.cnr.it, dario.disilvestre@itb.cnr.it

Plant growth-promoting bacteria (PGPR) interact with plants improving plant fitness, development, and responses to environmental stresses. In this scenario, the rhizobacterium *Pseudomonas simiae* is one of the most characterized PGPR, and it can colonize *Arabidopsis thaliana* roots thus exerting beneficial effects on plant nutrition, with the possible involvement of formate dehydrogenase (FDH). To get insights on plant-rhizobacteria interactions, roots of *A. thaliana* wt Col and of FDH knock-out mutant plants grown in soil were therefore exposed to *P. simiae* WCS417. Leaf proteome was extracted from treated and control plants, and the physiological modulations were investigated. Leaf proteome changes upon root exposure to *P. simiae* were more pronounced in the FDH mutant than in wt. The characterized proteomes were also evaluated by computational approaches in the context of systems biology. In particular, protein-protein interaction (PPI) network models were reconstructed and processed at topological level to shed light on the central role of some proteins, called hubs, in stress-responsive pathways and the metabolic processes activated by beneficial plant-bacteria interaction.

Keywords: *Arabidopsis thaliana*, Proteomics, *Pseudommonas simiae* WCS417, Rhizobacterium, PPI, Systems Biology.

Multi-Omics driven identification of yield limiting factors in Molecular Pharming.

Nicholas Prudhomme[4], Rebecca Pastora[1], Mike D. McLean[1], Doug Cossar[1], Jonathan Krieger[2], Amanda Sproule[3], David Overy[3], Jennifer Geddes-McAlister[4]

[1] PlantForm Corporation (Toronto, Canada)
[2] Bruker Daltonics (Toronto, Canada)
[3] Agriculture and Agri-Food Canada (Ottawa, Canada)
[4] University of Guelph (Guelph, Canada)

Email corresponding author: nprudhom@uoguelph.ca

Plant-based production systems for biopharmaceutical proteins provide an attractive alternative to mammalian cell, yeast, or bacterial systems. Benefits of this system include a reduced cost of drug development, improved scalability, rapid delivery of new products to the market, and an ability to provide safe and efficacious medicines for various diseases. Although the plant-based production platform offers excellent potential for therapeutic protein production, barriers still exist that impact the product yield and limit wider adoption of the technology. Challenges regarding purity and abundance of target proteins produced by agroinfiltration include host defense responses and unintended proteolysis, which restrict transition of the technology and products for industrial use. Here, we use tandem mass tag systems combined with state-of-the-art mass spectrometry-based proteomics to investigate the plant defense response, profile the changes to bacterial invasion during infection and how the interaction between host and pathogen changes throughout the infection process. In this project, we infiltrated *Nicotiana benthamiana* plants with *Agrobacterium tumefaciens* grown in bioreactor or shake flask conditions and collected leaf tissue at 0, 1, 3 and 7 dpi. Thereafter, proteins were extracted and subjected to mass spectrometry on an orbitrap tribrid mass spectrometer and identified over 2500 plant proteins and 350 bacterial proteins. In addition, the metabolome was profiled to integrate the impact of infection at multiple molecular levels. Data analysis reveals culture- and time-dependent responses to infection, along with optimal target protein production. We also observed differences in the plant defense response system based on culture conditions of the bacterium and uncover novel biological insight into the relationship between the host and pathogen. Together, our research enhances the molecular pharming pipeline and supports innovative applications of the technology.

Keywords: Molecular pharming, proteomics, metabolomics, systems biology, *Agrobacterium tumefaciens*, *Nicotiana benthamiana*, bioreactor, shake flask, defense response, antibodies.

A multi-omic framework of calcium-mediated kiwifruit ripening.

Chrysanthi Polychroniadou[1,2], Michail Michailidis[2], Ioannis Adamakis[3], Christina Skodra[1], Ioannis Ganopoulos[2], Martina Samiotaki[4], Christos Bazakos[2], Evangelos Karagiannis[5], Georgia Tanou[2], Athanassios Molassiotis[1]

[1]Laboratory of Pomology, Department of Horticulture, Aristotle University of Thessaloniki, Thessaloniki 57001, Greece
[2]Joint Laboratory of Horticulture, Institute, Thessaloniki, Greece
[3]Department of Botany, Faculty of Biology, Athens, Greece
[4]Institute for Bioinnovation, Biomedical Sciences Research Center "Alexander Fleming", Vari 16672, Greece
[5]Department of Agriculture, University of Western Macedonia, 53100 Florina, Greece

Email corresponding author: polychry@agro.auth.gr

Kiwifruit postharvest ripening has been addressed to be related to calcium (Ca), however, the underlying mechanisms by which Ca regulates this process remain largely unexplored. In the current study, 'Hayward' kiwifruits were harvested commercially and dipped in 2% $CaCl_2$. Afterward, the exposure to cold storage (0 °C, RH 95%) for 3 months and the subsequent ripening traits of fruit at 20°C for 7 days was evaluated. Data indicated that Ca-treated kiwifruits exhibited higher endogenous Ca concentration and signals of fluorescent calcium indicator Fluo3, in the outer pericarp and placenta of kiwifruit. Also, Ca-treated fruit exhibited an increase in firmness and a decrease in ethylene production. Fluorescent microscopy with cell wall primary antibodies showed that de-esterified homogalacturonans (HG) (antibody LM19) increased in Ca-treated fruit in contrast to arabinogalactan proteins (AGPs) (antibodies LM13 and LM30) that their detection levels were down accumulated. Metabolomic analysis revealed 27 primary (i.e. glucolic acid, oxoproline and galactinol) and 4 secondary (i.e. catechin, epicatechin, procyanidin B2) metabolites that altered in response to Ca. RNA-seq analysis for early (12 hours after dipping) and late response (after 3 months of cold storage) to Ca highlighted 89 and 370 differentially expressed genes, respectively, mainly involved in ethylene, calcium and cell wall metabolism. Also, proteomic analysis revealed a high number (890) of proteins that were affected by Ca, notably during the late period. Weighted correlation and network analysis based on the integration of transcriptome, metabolome, and proteome datasets identified candidate modules involved in the Ca postharvest action in kiwifruit. Following global correlation analysis of proteome and transcriptome levels we found a positive correlation value for all pairwise comparisons in Ca-treated kiwifruit. These results provide basic information regarding the Ca function in fruit ripening, which may be helpful for kiwifruit postharvest control.

Keywords: Calcium, kiwifruit, metabolomics, proteomics, ripening, transcriptomics.

The mystery of the standard system: Stand-alone activity or proton transporting redox chain?

Sabine Lüthje[1], Matthias Menckhoff[1], Mandy Graff[1], Lara-Marie Hallscheid[1], Ivana Boris[1], Sarah Kienscherf[1], Sönke Harder[2]

[1]Plant Proteomics and Oxidative Stress Group, Department of Biodiversity of Crop Plants, Universität Hamburg, 22609 Hamburg, Germany
[2]Center for Diagnostics Clinical Chemistry and Laboratory Medicine, Core Facility of Mass Spectrometric Proteomic, Campus Forschung N27, University Hospital Hamburg-Eppendorf (UKE), 20246 Hamburg, Germany

Email corresponding author: s.luthje@plantphysiology.de

Fourty years ago, a transmembrane ferricyanide (HCF III, hexacyanoferrate III) reductase activity, the so-called standard system, was discovered in animal and plant plasma membranes. Application of HCF III caused a depolarization of the membrane potential, a reduction of the electron acceptor and an increased proton secretion. Biochemical data suggested NAD(P)H as the natural electron donor of this constitutive redox system. The observed *in vivo* HCF III reductase activity was stimulated by K-type quinones, whereas vitamin K antagonists (e.g. dicumarol) or tenoyltrifluroaceton inhibited the transmembrane redox activity. These obseravations, together with the identification of vitamin K_1 in plasma membranes, suggested involvement of the quinone in transmembrane electron transfer. Here a systems biological approach was used to unravel the mystery of the molecular structure and physiological function of the standard system. *In vitro* experiments with NAD(P)H-loaded plasma membrane vesicles verified NAD(P)H as the primary electron donor for the observed trans-membrane redox activity. Besides vitamin K_1, several *b*-type cytochromes and flavoproteins were identified in plasma membranes of maize (*Zea mays* L.) roots. Interaction of these compounds with different effectors was investigated by dye-affinity chromatography combined with mass spectrometry and biochemically characterization of the partially purified proteins. *In silico* analyses of the identified redox compounds verified binding-sites for quinones, coumarins and diphenyleniodonium, and a function in oxidative stress response. These data supported the existence of a quinone mediated electron transport chain in the plasma membrane and its function in apoplastic redox homeostasis.

Keywords: coumarin, DPI, plasma membrane, redox proteome, vitamin K, *Zea mays*.

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The Proteomes that Feed the World.

Guido Giordano[1], Sarah Brajkovic[2], Qussai Abbas[3], Paula Andrade-Galan[3], Ezgi Aydin[3], Gianluca Corongiu[3], Genc Haljiti[3], Sophia Hein[3], Jiuyue Pan[3], Mario Picciani[3], Andrea Piller[3], Veronica Ramirez[3], Patrick Roehrl[3], Cemil Saylan[3], Armin Soleymaniniya[3], Sebastian Urzinger[3], Lukas Wuerstl[3], Claus Schwechheimer[1], Brigitte Poppenberger[4], Bernhard Kuster[2]

[1]Chair of Plant Systems Biology; Technical Universitiy Munich. Freising, Germany
[2]Chair of Proteomics and Bioanalytics; Technical University of Munich, Freising, Germany
[3]School of Life Sciences Weihenstephan. Technical Universitiy Munich. Freising, Germany
[4]Professorship of Biotechnology of Horticultural Crops; Technical University of Munich, Freising, Germany

Email corresponding author: guido.giordano@tum.de

Plants represent the nutritional basis of virtually all life on Earth. Protein-rich foods from crop plants are essential for sustaining an increasing human population and counteracting climate change. While an increasing number of crop genome sequences are being elucidated, little is known about their proteomes - the entirety of proteins that orchestrate cellular functions. In an effort to fill this knowledge gap, we assembled the “Crop Proteome Engine”: a team of young and experienced scientists with leading expertise in plant science, proteomics and bioinformatics, supported by a large international group of excellent partners. Our goal is to map the proteomes of all major tissues and organs of the 100 most important crop plants for human nutrition. To support a plant proteomic project of this scale, a high-throughput proteomic workflow was devised. We combined a universal protein extraction method with an automated one-pot sample preparation protocol (SP3) on a liquid handling robot. In addition, we provided an automated off-line peptide separation protocol using an AssayMap Bravo liquid handling platform and optimized micro-LC-MS/MS conditions. Protein identification and quantification was performed by MaxQuant and Prosit rescoring. This method already succeeded in defining with high efficiency, reproducibility and coverage the proteome of several crops, including quinoa (*Chenopodium quinoa)*, sorghum (*Sorghum bicolor)*, potato (*Solanum tuberosum)* and tomato (*Solanum lycopersicum)*, enabling the identification and quantification of >10,000 proteins from any plant tissue within six hours of instrument measurement time. We are still looking for partners interested in joining the initiative by contributing plant material or bioinformatics capabilities. We expect that the creation of this first crop proteome atlas, which will be made publicly available on PRIDE and ProteomicsDB and which will be enriched by several relevant research projects, will generate leverage points for innovations in crop production, food processing and the molecular understanding of plant biology.

Keywords: nutritional plant proteomics, liquid chromatography, mass spectrometry.

Acknowledgment: this research was in part funded by the Elitenetzwerk Bayern.

DEVELOPMENTAL PROTEOMICS

Single-cell type proteomics and metabolomics: new insights into guard cell immunity and CO_2 response.

Sixue Chen[1]

[1]Department of Biology, University of Mississippi, Oxford, USA

Email corresponding author: schen8@olemiss.edu

Human population is expected to reach 9 billion by 2050, and global crop productivity needs to increase by 70% to feed the growing population. Unfortunately, pathogen infection and other adverse environmental conditions have posed grand challenges to crop yield and food security. Stomatal pores are major entry points of bacteria pathogens. How stomatal guard cells respond to pathogen invasion and other environmental factors (e.g., rising CO_2 levels) is an important and interesting question. Recently, we have reported a new redox proteomics method called cysTMTRAQ that combines two types of isobaric tags, isobaric tag for relative and absolute quantification (iTRAQ) and cysteine tandem mass tag (cysTMT) in one experiment. The method not only enables simultaneous analysis of cysteine redox changes and total protein level changes, but also allows the determination of bona fide redox modified cysteines in proteins through correction of protein turnover. This technology has recently been applied to discover potential redox proteins in stomatal guard cells in response to the flagellin's N-terminal domain's 22-aa peptide (flg22) of *Pseudomonas syringae* pv. tomato str. DC3000 (PstDC3000). Stomatal closure was observed within 5 minutes of the flg22 treatment and became significant after 15 minutes of treatment. Reactive oxygen species (ROS) levels increased throughout the time course of treatment, and reached the peak at 15 minutes. Based on these results, three time points (15, 30 and 60 minutes) were selected for the cysTMTRAQ experiments. A total of 2144 proteins were identified, 677 contained cysteines with cysTMT labels, and 57 showed significant redox changes ($q < 0.05$) after flg22 treatment. Here, the functional characterization of a lipid transfer protein in guard cell innate immunity is reported. As CO_2 levels affect stomatal immunity and stomatal movement, we studied CO_2 signaling using hyphenated metabolomics technologies. A new signaling pathway involving jasmonic acid was discovered. Future directions in signal crosstalk and data integration in the context of stomatal disease triangle will be discussed.

Keywords: *Arabidopsis thaliana*, proteomics, metabolomics, guard cell, immunity, CO_2.

Multilevel regulation of seed dormancy and germination in hexaploid wheat revealed through proteogenomic, redox proteomic, and biochemical approaches.

Natalia V. Bykova[1], Nataša Radovanovic[1], Mei Huang[1], Michelle Rampitsch[1], Zhen Yao[1], M. K. Pabasari S. Wijesinghe[1,2], Junjie Hu-Skrzenta[1,2]

[1]Morden Research and Development Centre, Agriculture and Agri-Food Canada, Morden, MB, R6M 1Y5, Canada
[2]Department of Biology, Memorial University of Newfoundland, St. John's, NL, A1C 5S7, Canada

Email corresponding author: Natalia.Bykova@agr.gc.ca

Seed dormancy control in wheat is complex, and genetic factors responsible for it are dispersed on almost every wheat chromosome. Consistent with the genetic complexity is wide range of physiological and physical attributes affecting dormancy. Generation of reactive oxygen species (ROS), antioxidative systems, phytohormonal regulation and their reciprocal signaling play important roles in releasing embryo cells from the quiescent state during seed dormancy removal. In this study, proteins and genes essential to regulation of dormant or high germination potential physiological states, ROS-mediated modification of redox responding proteins, and antioxidant systems involved in governing complex seed dormancy trait were revealed. Hard white spring wheat (*Triticum aestivum* L.) doubled haploid population and integration of proteogenomic, pharmacological, quantitative thiol-specific redox proteomic, and biochemical approaches were employed. In dormant embryos, phenotype-specific alterations were found for proteins involved in modulation of ROS in cell wall, extracellular compartment, and mitochondria. Proteomic signatures of dormancy phenotype consisted of signaling proteins associated with flowering, downstream targets of ABA-dependent germination repression pathway, enzymes responsible for alteration of intracellular ABA levels and structural integrity of plant cell wall. Induction of early germination in embryos was accompanied by higher capacity for the provision of NADPH reducing equivalents for biosynthetic processes, generation of lipid second messengers, protein targeting to 26S proteasome, positive modulation of GA signaling, and regulation of osmosensory responses. Inhibition of ROS production in the presence of ABA had synergistic effect on blocking germination in non-dormant seeds. Our results demonstrate that germination induction is mediated through the redox control, and antioxidative pathways in wheat embryos are involved in dormancy regulation. The total glutathione levels were significantly higher in dormant than in non-dormant embryos, whereas the total ascorbate levels increased upon dormancy release indicating high capacity for ascorbate regeneration and different roles of ascorbate and glutathione in regulation of seed germination.

Keywords: iTRAQ, iodoTMT, RNA-Seq, seed dormancy, germination, *Triticum aestivum*.

Acknowledgment: This work was supported by the Agriculture and Agri-Food Canada funding to NVB (AAFC Agri-Innovation Program, projects J-001356 and J-002600).

Arabidopsis PP6-type phosphatase complex: a missing link between strigolactone and auxin signaling?

Sylwia Struk[1,2], Dan Chen[1,2], Arne Temmerman[1,2], Anse Jacobs[1,2], Lam Dai Vu[1,2], Evelien Mylle[1,2], Annick De Keyser[1,2], François-Didier Boyer[3], Geert Persiau[1,2], Dominique Eeckhout[1,2], Daniel Van Damme[1,2], Geert De Jaeger[1,2], Ive De Smet[1,2], Kris Gevaert[4,5], Sofie Goormachtig[1,2]

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium,
[2]VIB, Center for Plant Systems Biology, 9052 Ghent, Belgium,
[3]Institut National de la Recherche Agronomique, Institut Jean-Pierre Bourgin, Versailles, France
[4]Center for Medical Biotechnology, 9052 Gent, Belgium
[5]Department of Biomolecular Medicine, Ghent University, 9052 Gent, Belgium.

Email corresponding author: sylwia.struk@psb.vib-ugent.be

Strigolactones (SLs) regulate multiple aspects of plant growth and development, such as shoot and root architecture, through ubiquitination and subsequent degradation of the repressor proteins SUPPRESSOR OF MORE AXILLARY GROWTH 1 (SMAX1)-LIKE (SMXL) 6, SMXL7, and SMXL8. Other members of this protein family, SMAX1 and SMXL2, act in the pathway of the chemically related karrikins (KAR), presumed to mimic still unknown endogenous compound(s), regulate mainly seed germination, and seedling photomorphogenesis. Although SMXL proteins play critical role in a wide range of developmental processes in plants, their exact mode of action has not yet been fully elucidated. While they undoubtedly control transcriptional responses, some of their functions are transcription independent and might be carried out by the interaction with different adapter proteins. These transcription-independent responses involve modulation of auxin flow by affecting PIN-FORMED1 (PIN1) auxin efflux carrier localization, abundance, and trafficking at the membrane. In the present study, we identified Protein phosphatase 6 (PP6)-type complex as a novel interactor of SMXL7 using tandem affinity purification in *Arabidopsis thaliana* cell cultures. The role of PP6 complex has already been described in the signaling pathway of ABA, light and auxin transport, by the dephosphorylation of PIN proteins. Our biochemical data confirmed that not only SMXL7 but also SMAX1 physically associates with PP6-type complex. In agreement, using genetic analysis we demonstrated that the PP6 complex might regulate many SL- and KAR-related developmental processes, but does not affect the expression of known SL and KAR marker genes. The PP6 complex seems not to affect the phosphorylation status of SMXL7, as well as its localization to the nuclear condensates, transcript levels, and protein stability. Altogether, our data suggest that PP6-type complex might be a part of transcription-independent responses downstream of SMXL proteins, possibly by dephosphorylating one of its known targets, for instance, PINs, or those that are yet to be discovered.

Keywords: strigolactones, karrikin, PIN proteins, affinity purification, phosphorylation.

The cell wall proteome of an early land plant, Marchantia polymorpha: conserved protein families and specific ones in relation to its cell wall composition.

Hasan Kolkas[1], Josiane Chourré[1], Thierry Balliau[2], Michel Zivy[2], Hervé Canut[1], Elisabeth Jamet[1]

[1]Laboratoire de Recherche en Sciences Végétales, Université de Toulouse, CNRS, UPS, Toulouse INP, F-31320 Auzeville-Tolosane 31320, France
[2]Université Paris-Saclay, INRAE, CNRS, AgroParisTech, GQE - Le Moulon, PAPPSO, F-91190 Gif-sur-Yvette, France

Email corresponding author: elisabeth.jamet@univ-tlse3.fr

Typical components of plant cell walls include cellulose, hemicelluloses, and pectins as well as cell wall proteins (CWPs). The CWPs are implicated in cell wall polymers remodeling and signaling during development and in response to environmental cues. In this study, the cell wall proteome of an early land plant, *Marchantia polymorpha*, is described for the first time. *M. polymorpha* is attracting attention as a new model because of its particular position in the green lineage, its easy genetic transformation and its small-sequenced genome. Two complementary strategies were used: extraction of proteins from purified cell walls and affinity chromatography of a total protein extract on Concanavalin A to capture *N*-glycoproteins. Three developmental stages were studied after the transfer of gemmae to fresh culture medium: 2 weeks (transition to thallus shape), 3 weeks (regular growth) and 5 weeks (appearance of gemma cups). LC-MS/MS analysis and bioinformatics allowed the identification of 410 proteins with predicted signal peptides and assumed to be *bona fide* CWPs. They represented around one-third of the expected cell wall proteome. The proportion of proteins acting on cell wall carbohydrates was slightly lower than in previously described flowing plants cell wall proteomes, whereas that of oxido-reductases, like class III peroxidases and polyphenol oxidases, was higher. Similarly, dirigent proteins were more represented in correlation with the presence of high amounts of phenolic compounds. Remarkably, six pectin methylesterases were identified, consistently with the presence of demethylated homogalacturonans (HGs) and very low levels of methylated HGs as revealed by specific monoclonal antibodies on polysaccharide arrays. Seven D-mannose lectins were identified, in agreement with the presence of a high mannan content as detected with antibodies. Finally, a quantitative approach showed the dynamics of the cell wall proteome during the development of thalli.

Keywords: *Arabidopsis thaliana*, cell wall, cell wall polysaccharides, *N*-glycoproteome, phenolic compounds, proteomics.

Acknowledgment: Work supported by the TULIP LabEx (contract ANR-10-LABX-41; ANR-11-IDEX-0002-02).

Cell-type resolution proteomics and metabolomics for functional annotation of the grain filling process in wheat endosperm.

Shuang Zhang[1,5], Arindam Ghatak[1], Mitra Mohammad Bazargani[2], Prasad Bajaj[3], Rajeev K Varshney[4], Dong Jiang[5], Palak Chaturvedi[1*], Wolfram Weckwerth[1,6*]

[1]Molecular Systems Biology Lab (MOSYS), Department of Functional and Evolutionary Ecology, University of Vienna, Djerassiplatz 1, 1030 Vienna, Austria
[2]Agriculture Institute, Iranian Research Organization for Science and Technology, Tehran, Iran
[3]Centre of Excellence in Genomics and Systems Biology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, 502324 India
[4]State Agricultural Biotechnology Centre, Centre for Crop and Food Innovation, Food Futures Institute, Murdoch University, Murdoch, WA, 6150, Australia
[5]National Technique Innovation Center for Regional Wheat Production/Key Laboratory of Crop Ecophysiology, Ministry of Agriculture/Nanjing Agricultural University, Nanjing 210095, Jiangsu, China
[6]Vienna Metabolomics Center (VIME), University of Vienna, Djerassiplatz 1, 1030 Vienna, Austria

Email corresponding authors: palak.chaturvedi@univie.ac.at, wolfram.weckwerth@univie.ac.at

Grain development is a key stage in the life cycle of many plants. The development of seeds is the basis of agriculture and the primary source of calories consumed by humans. Here, we employed laser micro dissection (LMD), combined with shotgun proteomics and primary metabolomics, to generate a cell-type proteome and metabolome atlas of developing wheat endosperm at the early and late grain filling stages. We identified 1803 proteins from four different cell layers (aleurone (AL), sub-aleurone (SA), starchy endosperm (SE), and endosperm transfer cells (ETCs)) of developing endosperm 15 days after anthesis (DAA) and 26 DAA. Sixty-seven differentially expressed proteins in the AL, 31 in the SA, 27 in the SE, and 50 in the ETCs were detected between these two time points. We observed high general metabolic activity of the grain regarding carbohydrate metabolism, defence against oxidative stress, and signalling in the different cell layers during the grain filling process. Forty-two metabolites were identified and performed a distinct metabolite profile in different developing endosperm cells at early and late grain filling stages. Based on these data, we propose a model for sugar loading and starch biosynthesis in developing wheat grain endosperm. Cell-specific identification of SUT and GLUTsugar transporters, sucrose converting and starch biosynthesis enzymes suggest a grain filling model via nucellar projections and endosperm transfer cells (ETCs) in the early stage initiating starch biosynthesis in the starchy endosperm (SE). This is followed by a functional switch of the proteome from the early to late grain filling stage.

Keywords: Wheat, proteomics, aleurone, sub aleurone, starchy endosperm, transfer cells, laser micro dissection (LMD).

Acknowledgment: Work supported by the China Scholarship Council (CSC) (Grant number: 201706850037) and completion grant of the Vienna Doctoral School Ecology and Evolution of the Faculty of Life Sciences, University of Vienna, Austria. We are also thankful to the European Union Horizon 2020 research and innovation programme under grant agreement number GA 2020 862-858 (ADAPT) abd the Austrian Science Fund (FWF, DerWissenschaftsfonds), Grant agreement number I 5234 for their support.

TECHNICAL/ METHODOLOGICAL ADVANCES IN PROTEOMIC ANALYSIS

Fruit-omics: from the 2DE era, over shotgun MS, towards single cell omics.

Sebastien Carpentier[1]

[1]Alliance of Bioversity International and International Center for Tropical Agriculture (CIAT)

Email corresponding author: s.carpentier@cgiar.org

For a healthy and sustainable food production and to combat the effects of climate change, we depend on biodiverse crop systems. In-depth knowledge on the added value of our previous biodiversity is therefore of utmost importance. However, in many (fruit) crops there is a big knowledge gap. In the light of crop improvement, we intend to fill this gap by linking phenotyping to different –omics techniques. Over the years, technology evolved and strategies adapted. Two-dimensional gel electrophoresis in combination with de novo MS was originally the only option. It was a powerful technology that was mainly limited in its throughput. But as soon as the first draft genomes came out, the way was paved for a gel free shot gun approach in combination with transcriptomics. International consortia were formed to exchange knowledge and to stimulate the integration of different omics techniques with phenotyping. Our on line phenotypical approaches have enabled us to bring forward new hypothesis and reveal unique allele specific proteins. With the development of new bio-informatics/statistics techniques, a real integration with all omics also became possible in various fruit species. With the development of new sample prep techniques and more sensitive mass spectrometers, the so called “single cell omics” became into the picture. In view of more water use efficient sustainable production systems, we are currently focusing on guard cell omics. Guard cells, controlling stomatal aperture, form the first and crucial boundary between the plant and the atmosphere. These cells are judges who determine carbon gain and water losses and have a huge influence on plant growth and development. The integration of this cell specific omics start shedding light in many different species.

Keywords: Phenotyping, omics integration, fruit, allele specific protein.

Identifying endogenous rust peptides using top-down peptidomics, de novo sequencing and bioinformatics strategies.

Christof Rampitsch[1], Slavica Djuric-Ciganovic[1], Mei Huang[1], Zhen Yao[1], Mark Lubberts[2], Peter Verhaert[3]

[1]Agriculture and Agrifood Canada, Morden Research and Development Centre, Morden MB, Canada.
[2]Agriculture and Agrifood Canada, Summerland Research and Development Centre, Summerland BC, Canada.
[3]Proteoformix, Beerse, Belgium.

Email corresponding author: chris.rampitsch@agr.gc.ca

Puccinia triticina (*Pt*) is an obligate fungal parasite that causes leaf rust on wheat. This disease occurs annually and potentially results in large yield losses. Host-pathogen communication at the protein level has been well-studied in this and similar pathosystems, but the potential roles of peptides (smaller than 5 kDa) have not been examined. This research describes a new project aimed at investigating the role(s) of peptides in this plant-pathogen interaction, using top-down LC-MS analyses to detect novel, intact peptides. For the LC-MS approach, peptides were initially obtained from rusted wheat leaves by precipitation from acetone, followed by enrichment using RP- and SEC-HPLC. Peptides were left intact and then analyzed by LC-MS in a high-resolution Orbitrap mass spectrometer to detect intact peptides. Correct identification through homology-based matching using Mascot and Maxquant required us to construct new *Pt* databases containing hypothetical peptides down to 10 amino acids in length, as such small peptides have been observed and shown to be translated from small open reading frames in other fungi. More recently, we have adopted a *de novo* sequencing approach which does not require any databases. We have used an N-terminal tryptic peptide enrichment strategy that improved our yield of endogenous peptides, and we are currently devising strategies to determine where on the rust genome these peptides are encoded.

Keywords: *Puccinia triticina*, peptidomics, short open reading frames, de novo sequencing.

Acknowledgment: Work supported by an internal grant from AAFC.

Success and bottlenecks of large scale plant protein turnover measurement: a tomato fruit maturation case study.

Willy Bienvenut[1], Marie Helene Andrieu[2], Olivier Langella[1], Sarah Naudin[2],Thierry Bailliau[1], Sophie Colombie[2], Mélisande Blein-Nicolas[1]

[1]Université Paris-Saclay, INRAE, CNRS, AgroParisTech, GQE - Le Moulon, PAPPSO, 91190, Gif-sur-Yvette, France
[2]Université de Bordeaux, INRAE, Biologie du Fruit et Pathologie, UMR 1332, Bordeaux Metabolome, MetaboHUB, PHENOME-EMPHASIS, 71 av E. Bourlaux, 33140 Villenave d'Ornon, France.

Email corresponding author: willy.bienvenut@universite-paris-saclay.fr

Proteostasis is a key element among the molecular mechanisms required to maintain the equilibrium between protein synthesis and degradation during organism's development. The protein turnover rate (PTR) is the parameter to survey to better understand proteostasis mechanisms which are associated to protein synthesis (K_s) and degradation (K_d) constants. Presently, PTR measurement based on pulse SILAC labelling are not compatible with autotroph species. Therefor, we developed a novel large scale proteomics strategy based on suboptimal ^{15}N metabolic labelling to calculate K_s and K_d constants in plants. Our approach essentially requires to determine the "protein fold change" (PFC) and the "protein labelled fraction" (PLF) at several labelling kinetics time points. These two values are calculated from mass spectrometry data after adapting MassChroQ, a peptide quantification software and MCQR, an R package dedicated to the statistical analysis of proteomics quantification. First, our developments take into account the peptide ^{15}N-modified isotopic distribution to perform robust label-free quantitation to determine the PFC for each characterised peptide/protein. Second, we used the variation of the peptide isotopic distribution to estimate the LPF and its evolution during the ^{15}N kinetic assay. Finally, we filtered the calculated K_d constants using statistical approaches. We tested this processing pipeline in a project dedicated to study tomato fruit growth and maturation. To this end, we first setup a methodology for tomato fruit ^{15}N labelling. Then we prepared and analysed the collected samples at different time points after the ^{15}N starting pulse. Finally, we used the raw mass spectrometry data to test our processing pipeline. This initial application provides an excellent opportunity to improve our processing pipeline and the results, successes and bottlenecks will be detailed more extensively during this presentation.

Keywords: Protein synthesis, Protein degradation, metabolic labeling, ^{15}N stable isotope, bioinformatics, MassChroQ, MCQR

Acknowledgment: Work supported by the INRAE BAP department (PROOFER grant). The proteomics analysis were performed on the PAPPSO facility which is supported by INRAE, the Ile-de-France regional council, IBiSA and CNRS.

Research using Data Independent or Data Dependent Acquisition in proteomics: selected applications.

Martina Samiotaki[1]

[1]Proteomic Facility, B.S.R.C. "Alexander Fleming", Vari, Attica, Greece

Email corresponding author: samiotaki@fleming.gr

Switching between the two major data acquisition methods DDA and DIA is one of the current dilemmas in running proteomic projects. The availability of modern and suitable instrumentation as well as the continuous development of data analysis tools is making the proteomic analyses more robust as well as more sensitive. Selected strategies using label-free proteomics with DIA for the generation of spatiotemporal data, interactomes and differential expression of proteomes will be presented. The more traditional DDA available protocols of analysing posttranslational modifications in our facility will also be discussed as well as our metaproteomics strategies, aiming to generate an overview of the arsenal technologies available B.S.R.C. "Al. Fleming".

Keywords: DIA, DDA, Label-free Quantification, Interactomes, Metaproteomics, Post translational modifications.

Variation in histone patterns with respect to plant developmental plasticity.

Gabriela Lochmanová[1,2], Pavlína Pírek[1,2], Zbyněk Zdráhal[1,2]

[1]Mendel Centre for Plant Genomics and Proteomics, Central European Institute of Technology, Masaryk University, CZ-62500 Brno, Czech Republic
[2]Faculty of Science, Masaryk University, CZ-62500 Brno, Czech Republic

Email corresponding author: gabriela.lochmanova@ceitec.muni.cz

A high degree of developmental plasticity enables plants to adapt to continuous, often unfavorable and unpredictable changes in their environment. At the molecular level, adaptive advantages for plants are primarily provided by epigenetic machinery. A wide range of biochemical and molecular biological approaches have been contributing to deciphering the machinery of epigenetic regulations. The scope of knowledge in the field of histone epigenetics is determined by the feasibility of respective analyses, which are challenging due to the complex pattern of histone post-translational modifications (PTMs) and the presence of histone variants. In this respect, proteomics offers unrivaled opportunities for in-depth investigation of histone function in the regulation of gene expression and cell fate. The outcome of mass spectrometry-based histone analysis is highly dependent on an appropriate sample preparation, which must be adapted for an individual purpose. To ensure the identification of the high number of histone variants and complex combinatorial pattern of their PTMs, chemical derivatization of amine groups in the protein sequences using propionic anhydride was performed, followed by labeling of newly released amine groups at peptide N-termini after trypsin digestion. To remove plant-species-specific contaminants and chemical additives incompatible with MS-based procedures, an optimized filter-aided sample preparation protocol was applied. Mass spectrometry-based proteomics allowed us to examine 1) inter-individual variability in levels of histone peptide forms in two *A. thaliana* ecotypes, 2) the impact of genetic and chemical inhibition of histone deacetylases on histone PTM pattern in *Arabidopsis* plants, and 3) the role of histone sequence variants and PTMs in suppressing chromatin misassembly in *Arabidopsis* chaperone mutants. In total, we demonstrate that the maintenance of essential functions of each cell in a multicellular organism relies on the close connection between individual processes of chromatin assembly.

Keywords: *Arabidopsis thaliana*, epigenetics, histone variants, post-translational modifications, mass spectrometry.

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High aspect ratio pillar array columns for deep proteome profiling at moderate LC pump pressures.

Pantelis Kapetis[1], Jeff Op de Beeck[2], Natalie Van Landuyt[2], Joshua Silveira[3], David Bergen[3], Tabiwang Arrey[5], Romain Huguet[3], Xuefei Sun[4], Brandon Robson[4], Robert Van Ling[6], Paul Jacobs[2]

[1]Pantelis Kapetis, Metrolab S.A., 3 Lekkaki Street, 11524 Athens, Greece
[2]Thermo Fischer Scientific, Technologiepark-Zwijnaarde 82, B-9052 Gent, Belgium.
[3]Thermo Fischer Scientific, River Oaks Parkway 355, CA 95134, San Jose, USA.
[4]Thermo Fischer Scientific, Lakeside Drive 527, CA94085, Sunnyvale, USA.
[5]Thermo Fischer Scientific, Hanna-Kunath-Straße 11, 28199 Bremen, Germany
[6]Thermo Fischer Scientific, Takkebijsters 1, Breda 4817BL, The Netherlands

Email corresponding author: robert.vanling@thermofisher.com

The quality of LC-MS based proteomics research relies to a large extent on the resolving power, scanning speed and sensitivity that HRAM mass spectrometers can provide to identify and quantify proteins with high confidence. The impact of the resolving power achieved with LC separation of enzymatically digested proteins must however not be underestimated. In search of increased separation power, LC column technology has been continuously evolving towards using smaller packing materials to present a continuous feed of peptides to the mass spectrometer. In this contribution, we report the evaluation of a novel type of pillar array column where the combination of reduced inter pillar distance and increased etching aspect ratio result in improved separation performance at moderate operating pressures. Chromatographic performance metrics were determined for different pillar array formats with varying pillar sizes. Digested Cytochrome C was injected with a Thermo Scientific Ultimate 3000 nanoRSLC instrument and detected using a 3 nL UV cell. Human proteome samples were analyzed using an Orbitrap mass spectrometer equipped with a FAIMS Pro interface. A dilution series (200, 500, and 1000 ng) was separated (300 nL/min) using non-linear solvent gradients (60-180 min). The resulting LC-MS/MS data were searched using Thermo Scientific Proteome Discoverer. Spectra were searched against the UniProt human database, peptide spectral matches were filtered to a 1% false-discovery rate using Percolator. Previously, we have reported on a novel generation of pillar array columns where pillar dimensions had been scaled down by a factor of 2 to increase resolving power. Even though separation performance was improved by a factor of up to 1.75, column permeability was decreased by a factor of 12. This seriously limited the range of flow rates at which columns could be operated, but also the maximum length at which they could be designed. By modifying the aspect ratio (AR, pillar height/inter pillar distance) of the separation bed, permeability could again be increased by a factor of 4, opening up opportunities to design LC columns with increased separation length and wider LC flow rate acceptances. Using a 2nd generation pillar array column with a length of 110 cm peak capacities (n_c=(T_G/FWHM)+1) up to 1600 could be obtained. In agreement with the UV based chromatographic performance evaluation, significantly more precursors could be characterized when performing single data-dependent LC-MS/MS analyses of a tryptic digest of a human cell line with FAIMS, namely near 20% more proteins groups and over 40% more unique peptides were identified. With LC-MS run times of 90, 120 and 150 min, we respectively identified 6521, 7165 and 7539 protein groups with 1000 ng of sample loaded on column. We will further evaluate the 2nd generation pillar array columns with optimized MS methods to maximize the proteome coverage within single LC-MS/MS analyses.

Highlight mass-spectrometry related innovations (30 words max)
Increases in nanoLC separation performance to facilitate comprehensive proteome coverage.

Keywords: µPAC Neo HPLC columns, Deep-diving proteomics, nanoLC-MS.

Development of strategies to decipher the extracellular peptidome.

Aurélie Dupriez[1], Thierry Balliau[2], Michel Zivy[2], Hervé Canut[1], Elisabeth Jamet[1]

[1]Laboratoire de Recherche en Sciences Végétales, Université de Toulouse, CNRS, UPS, Toulouse INP, F-31320 Auzeville-Tolosane 31320, France
[2]Université Paris-Saclay, INRAE, CNRS, AgroParisTech, GQE - Le Moulon, PAPPSO, F-91190 Gif-sur-Yvette, France

Email corresponding author: elisabeth.jamet@univ-tlse3.fr

Plant cell wall play critical roles during development and in response to environmental cues. In particular, they are involved in cell-to-cell signaling, and different types of molecules originated from cell wall components can become signals. Based on our expertise in cell wall proteomics, we are presently designing strategies to identify and characterize extracellular peptides or small proteins (<10 kDa) possibly involved in defense reactions. Two options have been investigated: (i) the analysis of extracellular fluids collected using low-speed centrifugation or (ii) the extraction of total peptides with different organic solvents. In both cases, special attention has been paid to limiting proteolysis by quickening the extraction process or adding protease inhibitors soon after the extraction. The selection of polypeptides smaller than 10 kDa has been obtained using a cut-off step, while the tryptic digestion has not been performed systematically. The results indicate that the two methods are complementary, some peptides being only found using either of them. Altogether, a whole range of peptides/small proteins has been identified in leaves of *Arabidopsis thaliana*. Moreover, it has been possible to define the native form of some signaling peptides as well as some post-translational modifications like the hydroxylation of proline into hydroxyproline (Hyp) residues. In the latter case, the variability of the Hyp pattern previously shown has been found again in SCOOP10, suggesting a role in the regulation of the biological activity of the peptides possibly through *O*-glycosylation.

Keywords: *Arabidopsis thaliana*, cell wall, extracellular fluid, peptidomics.

Acknowledgment: Work supported by the STRESS-PEPT ANR project (contract ANR-20-CE20-0025), the CNRS and the Paul Sabatier-Toulouse 3 University.

Contemporary proteomic strategies for cysteine modifications profiling in plants.

Jingjing Huang[1,2], Patrick Willems[1,2,3,4], Kris Gevaert[3,4], Joris Messens[5], Kate Carroll[6], Jing Yang[7], Frank Van Breusegem[1,2]

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052, Ghent, Belgium.
[2]VIB Center for Plant Systems Biology, VIB, 9052, Ghent, Belgium.
[3]Department of Biomolecular Medicine, Ghent University, 9000, Ghent, Belgium.
[4]VIB Center for Medical Biotechnology, VIB, 9000, Ghent, Belgium.
[5]VIB Center for Structural Biology, Vrije Universiteit Brussel, B-1050, Brussels, Belgium
[6]UF Scripps Biomedical Research, Department of Chemistry, Jupiter, FL, 33458, USA.
[7]State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing, 102206, China.

E-mail corresponding authors: Jingjing.huang@ugent.be, Patrick.Willems@psb.ugent.be

Protein cysteine residues containing reactive thiols are susceptible to oxidative post-translational modifications (Oxi-PTMs) that can affect protein function. Proteomic techniques that comprehensively profile these cysteine modifications are pivotal towards a better understanding of protein redox signaling. Recent technical advances in chemical tools and redox proteomic strategies have greatly improved selectivity, *in vivo* applicability, and quantification of cysteine modifications. Despite this substantial progress, still many challenges remain ahead. To get insight into cysteine-mediated redox switches in plants, we have generated an unprecedented view on the hydrogen peroxide-dependent cysteine sulfenic acid landscape using various proteomic approaches that inspired downstream redox studies. Our current work focuses not only on mapping of OxiPTM sites, such as cysteine sulfinic acids and hydrogen sulfide-mediated persulfides, but also on the quantification of relative or absolute OxiPTM levels in order to discern their physiological relevance in plants. Here, we will provide an update on the recent advances in proteomic strategies for cysteine modifications profiling, with emphasis on the progress we have made in *Arabidopsis* by using genetic and chemical proteomic approaches. We will further compare the advantages and disadvantages of current methods and discuss the outstanding challenges and future perspectives for plant redox research.

Keywords: Cysteine, thiol, oxidative post-translational modifications, proteomics.

Acknowledgment: Work supported by Research Foundation-Flanders (FWO) Postdoctoral fellowship (no. 1227020N to J.H., no. 12T1722N to P.W.).

Organomercury-based capture of S-nitrosated proteins by mass spectrometry in *Arabidopsis*.

Patrick Treffon[1], Elizabeth Vierling[1]

[1]Department of Biochemistry and Molecular Biology, University of Massachusetts Amherst, Amherst, MA, USA

Email corresponding author: ptreffon@umass.edu

S-nitrosation, the selective posttranslational modification of protein cysteine residues to form S-nitrosocysteine (-SNO), is one of the molecular mechanisms by which nitric oxide (NO) influences diverse biological functions and can alter protein function, interactions, and location. Despite the considerable biological importance of this posttranslational modification, significant gaps exist regarding the identification of this modification on a global scale. To investigate the *in vivo* nitrosoproteome of *Arabidopsis thaliana* we employed an organomercury-based approach that reacts directly, efficiently, and specifically with S-nitrosocysteine, enabling precise identification of S-nitrosocysteine–containing peptides and S-nitrosated proteins. Reaction of phenylmercury compounds with S-nitrosocysteine results in the formation of a relatively stable thiol–mercury bond. The method comprises three basic steps: (1) blocking reduced cysteines with methyl methanethiosulfonate (MMTS), (2) capture S-nitrosated proteins or peptides with *p*-aminophenylmercuric-acetate coupled to agarose beads, and release with mild performic acid, and (3) liquid chromatography/tandem MS analysis. The performic acid also oxidizes cysteine thiols to sulfonic acid, creating a unique MS signature that permits site-specific identification of the modified cysteines. We applied this method for the first time in plants, using floral tissues of an *A. thaliana* mutant lacking a central regulator of NO-homeostasis, S-nitrosoglutathione reductase (GSNOR, *hot5-2*). We identified 931 endogenously S-nitrosated proteins, including proteins previously described as targets for this kind of posttranslational modification, validating this new methodology. To date, this is the largest data set of S-nitrosated proteins reported.

Keywords: *Arabidopsis thaliana*, proteomics, S-nitrosation, S-nitrosylation, organomercury, GSNOR, *hot5-2*.

Acknowledgment: This research was funded by the NSF, grant number MCB-1817985.

THESSINPPO2023 - POSTERS

PLANT STRESS UNDER CLIMATE CRISIS

Comparative proteomics of Sugarcane smut fungus - *Sporisorium scitamineum* unravels dynamic proteomic alterations during *in vitro* lifestyle transitions.

Nalayeni Kumaravel[1], Cinzia Franchin[2,3], Leonard Barnabas Ebinezer[4], Ashwin, N.M.R.[1], Ilaria Battisti[4], Paolo Carletti[4], Anna Rita Trentin[4], Palaniyandi Malathi[1], Rasappa Viswanathan[1], Giorgio Arrigoni[2,3], Antonio Masi[4] and Ramesh Sundar A.*[1]

[1]Division of Crop protection, ICAR - Sugarcane Breeding Institute, Coimbatore, India.
[2]Proteomics Center of Padova University, Padova, Italy.
[3]Department of Biomedical Sciences, University of Padova, Padova, Italy
[4]Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova, Padova, Italy.

*Email corresponding author: rameshsundar_sbi@yahoo.co.in

The life cycle of *Sporisorium scitamineum* is a multistep process in which opposite mating type haploids (MAT-1 and MAT-2) fuse to develop into dikaryotic mycelia and subsequently infect sugarcane. Dimorphism is a crucial feature for the establishment of infection and fungal survival *in planta*. Proteomic studies have made significant contributions to the knowledge of metabolism and pathogenicity in several biological models. However, the life-cycle transition in *S. scitamineum* has not been explored using a proteomics approach so far. In the present work, an iTRAQ-based comparative proteomic analysis of haploids (MAT-1 and MAT-2), haploid to mycelial transition (24 hpc vs MAT-1 and 24 hpc vs MAT-2), and dikaryotic mycelial stage (48 hpc vs MAT-1 and 48hpc vs MAT-2) was carried out. Following LC-MS/MS and computational data analysis, 364 differentially abundant proteins representing the three stages were identified. Functional categorization and enrichment analysis indicated that in the dikaryotic mycelial stage there was an increased abundance of proteins related to tricarboxylic acid cycle and oxidative phosphorylation for ATP production, suggesting an induction of pathways associated with energy production and conversion. Furthermore, in the dikaryotic mycelial stage, metabolic processes of carbohydrate, amino acid, and lipid were predominantly altered compared to the haploid and haploid-to-mycelial transition phase. A large number of proteins directly related to the transcription and translation process also increased in abundance in mycelial and haploid-to-mycelial transition stages indicating that there could be significant reprogramming at the transcriptome and proteome in these stages. Additionally, several DAPs, especially in the dikaryotic mycelial stage were predicted as effector proteins. Taken together with the potential effectors, several stage-specific and dimorphic transition associated proteins identified herein are potential candidates for defective mutant screening to elucidate their functional role in the dimorphic transition in *S. scitamineum*.

Keywords: sugarcane, *Sporisorium scitamineum*, pathogen, infection, fungus.

Integrative proteomics to investigate the role of mitochondrial protease FTSH4 in *Arabidopsis* tolerance to long-term moderate heat stress.

Agata Maziak[1], Malgorzata Heidorn-Czarna[1], Hanna Janska[1]

[1]Department of Cellular Molecular Biology, Faculty of Biotechnology, University of Wroclaw, Wroclaw, 50-383, Poland

E-mail corresponding author: hanna.janska@uwr.edu.pl

The threat of global warming makes discovering plant tolerance mechanisms to long-term moderate heat stress particularly important. We previously reported that loss of the *Arabidopsis* mitochondrial protease FTSH4, exhibiting proteolytic and chaperone-like activities, reduces the plant thermotolerance to moderately high temperature (30 °C), but does not affect basal and acquired thermotolerance. To identify potential substrates and interaction partners of the FTSH4 protease unique to 30 °C and to gain insight into the changes in mitochondrial proteome of *ftsh4* compared to the wild-type in response to long-term 30 °C, we applied various proteomic approaches, such as co-immunoprecipitation withFTSH4-TRAP-FLAG coupled with mass spectrometry (MS), iTRAQ, COFRADIC, and the MS-based analysis of insoluble protein aggregates induced by heat. 109 mitochondrial proteins were identified by iTRAQ as being specific for moderately increased temperature, 17 by COFRADIC, and only 5 by co-immunoprecipitation, according to a comparison of the data obtained at 22 °C and 30 °C. In certain cases, proteins identified by one method at 30 °C were detected by a different method at 22 °C, e.g., 4 of the 17 proteins identified by COFRADIC at 30 °C were found by co-immunoprecipitation performed at 22 °C. Furthermore, the iTRAQ analysis revealed that the abundance of proteins identified by COFRADIC or co-immunoprecipitation either increases or decreases. In addition to the global analysis of integrative proteomics data, we will focus on a few specific proteins. For example, the adenine nucleotide transporter (ADNT1), which exchanges ATP for ADP across the inner mitochondrial membrane, is co-purified with FTSH4-TRAP-FLAG only at 30 °C. According to the iTRAQ analysis, this protein accumulates in *ftsh4* compared to the wild-type at both 22 °C and 30 °C; however, at 30 °C, in the absence of FTSH4, this protein forms detergent-insoluble aggregates. Together, this suggests that ADNT1 is a substrate for the chaperone activity of FTSH4, which prevents its aggregation.

Keywords: *Arabidopsis thaliana*, mitochondrial protease, thermotolerance to moderately high temperature, integrated proteomics.

Acknowledgment: Work supported by grant 2017/27/B/NZ2/00558 from the National Science Centre, Poland, and EPIC-XS, project number 823839, funded by the Horizon 2020 program of the European Union.

Metabolome and proteome changes of the wheat under drought treatment.

Pavel Vítámvás[1], Nešporová Tereza[1,2], Klára Kosová[1], Sophie Charton[3], Sebastien Planchon[3], Radovan Hynek[2], Ilja T. Prášil[1], Jenny Renaut[3]

[1]Crop Research Institute, Drnovska 507/73, 161 01 Prague, Czechia
[2]University of Chemistry and Technology, Technicka 5, 160 00 Prague, Czechia
[3]Luxembourg Institute of Science and Technology, Avenue des Hauts-Fourneaux 5, L-4362 Esch-sur-Alzette, Luxembourg

Email corresponding author: vitamvas@vurv.cz

Plants have evolved various strategies to cope with drought stress in relation to their other needs and processes such as the necessity to supply carbon for photosynthetic assimilation in plants with a C3 type of photosynthetic assimilation. The aim of the present study was to investigate differential responses of Baletka and Tobak as model cultivars representing the contrasting drought response strategies to short-term and long-term drought treatments and the following recovery after rewatering at the physiological, proteome and metabolome levels. Bread wheat cultivars Baletka as a representative of conservative water-saving strategy and Tobak as a representative of water-spending strategy, were analysed with respect to a-short-term (under controlled condition 19 °C, 16 h photoperiod, irradiance 350 μmol.m-2.s-1; 7-day treatment at 30% soil water capacity) and a long-term drought (additional 10-day treatment at 30% soil water capacity) and the following rewatering (3-day at 70% soil water capacity). Physiological characteristics clearly distinguished Baletka and Tobak. In water-saving Baletka, significant remodelling of cell wall, changes in endocytosis and cell signalling, and especially, changes in synthesis of other defence proteins and LTI65kDa protein (also known as RD29B protein) were revealed by proteome and metabolome analyses. Moreover, the enhanced accumulation level of oxalate oxidase implied enhanced oxaloxidase activity leading to faster degradation of oxalate with the simultaneous gain of two molecules of CO2. The enhanced oxaloxide activity may represent an alternative source of CO_2 for plants with C3 type of photosynthesis suffering under drought stress.

Keywords: *Triticum aestivum*, proteomics, metabolomics, water-saving, water-spending.

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The abundance of the Calvin–Benson–Bassham enzymes is not affected by growth at CO_2-enriched atmosphere in *Eucalyptus* young plants.

Amanda C Baldassi[1], Leticia R Moretto[1], Gennifer Merrihew[2], Julia Robbins[2], Michael J MacCoss[2], Tiago S Balbuena[1]

[1]Department of Agricultural and Environmental Biotechnology, São Paulo State University, Brazil
[2]Department of Genome Sciences, University of Washington, USA

Email corresponding author: tiago.balbuena@unesp.br

Eucalyptus plants play a major role in the Brazilian forest sector. Besides being the primary source for the production of pulp and paper, the fast-growing evergreen trees have a huge potential to assimilate and stock massive amounts of atmospheric carbon. We used proteomics as a multidimensional technology to understand the metabolic changes that occur in *Eucalyptus* leaves when young plants were cultivated in a high CO_2 environment. For this purpose, different *Eucalyptus* species were cultivated in plant growth chambers for 30 days at 420, 550 and 680 ppm of CO_2. Plant species that presented the most dissimilar phenotypic responses were *Eucalyptus pellita*, *Eucalyptus urophylla* and *Eucalyptus grandis* grown at 680 ppm of CO_2. In order to evaluate the biochemical responses of the Calvin-Benson (CB) cycle upon cultivation at high CO_2, a mass spectrometer data dependent acquisition approach (DDA) coupled to a label free quantification was carried out. In total, 29 proteoforms were confidently identified and all the 13 CB enzymes were inferred. Only one statistical difference was detected in the abundance of the FBPase enzyme in *E. urophylla* identified in leaves from plants cultivated at 680 ppm compared to those cultivated at 420 ppm. Validation of the DDA data was carried out through a data-independent acquisition (DIA) approach, in which 50 proteoforms were identified from the same pool of the 13 CB enzymes. Although no statistical difference was observed, a decreasing trend in the abundance of CB enzymes could be detected only in *E. grandis* plants grown at 680 ppm. Our data suggested that the high CO_2 atmosphere induced growth in young *Eucalyptus* plants even in the absence of an increase in the abundance of the enzymes involved in carbon assimilation.

Keywords: abiotic stress, chloroplast biology, climate change.

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Proteomic characterization of enzymatic-generated antifungal bioactive peptides originating from okara.

Giulia Ceravolo[1], Stefano De Benedetti[1], Chiara Magni[1], Davide Emide[1], Bhakti Prinsi[2], Luca Espen[2], Mattias Pasquali[1], Alessio Scarafoni[1]

[1]Department of Food, Environmental and Nutritional Sciences, University of Milan, 20133 Milan, Italy
[2]Department of Agricultural and Environmental Science, Production, Territory, Agroenergies, Universita degli Studi di Milano, 20122 Milan, Italy

Email corresponding author: alessio.scarafoni@unimi.it

Application projects often provide scientific information which, contrary to what is normally pursued, can constitute a starting point for understanding physiological and biochemical aspects that are still unclear and incomplete. This work fits into this context. Conversion of agri-food byproducts for the full use of natural resources is one of the main challenges faced nowadays by the industrialized world. Okara is a byproduct generated in huge quantities during soymilk or tofu production, thus posing a significant disposal problem. Okara has high protein content (about 25-40%, mainly seed storage proteins, SPs), which makes this byproduct interesting and exploitable from a biotechnological point of view. Antifungal peptides have been described in plant seeds and okara as well, indicating the potential to produce bioactives exploitable for integrated pest management. This work aims to describe a rapid and economic procedure to isolate proteins from okara, and to produce and characterize an enzymatic proteolyzed product, active against fungal plant pathogens. A dose-response inhibitory activity was established against fungi belonging to the *Fusarium* genus. Enrichment of the active fraction was obtained through the combination of different chromatographic techniques. Mass Spectrometry analyses allowed the identification of potential candidate bioactive peptides. The exploitation of okara to produce antifungal bioactive peptides has not only the potential to turn this by-product into a paradigmatic example of circular economy but also opens perspectives on the multifunctionality of SPs, allowing to pave the way for understanding the possible bioactivity of peptides that originate from the breakdown of protein reserves during germination.

Keywords: *Glycine max*, proteomics, seed, storage proteins, proteolysis.

Acknowledgment: Work supported by CARIPLO Foundation (Milan, Italy), Bando Economia Circolare 2018, grant number 2018–0983.

Pistachio adaptation to salt stress as revealed by physiological and proteomics studies.

Ramesh Katam[1], Mohammad Akbari[1,2], Rakesh K Singh[3], Dalia Vishnudasan[4], Mostafa Farajpour[5], Prashanth Survajhala[4]

[1]Department of Biological Sciences, Florida A&M University, Tallahassee FL USA
[2]Pistat Research Center, Nazari Business Group, Tehran, Iran
[3]Translational Laboratory, Florida State University, Tallahassee FL USA
[4]Amrita School of Biotechnology, Amrita Vishwa Vidhyapeetham, Clappana PO 690525, Kerala India
[5]Crop and Horticultural Science Research Department, Mazandaran Agricultural and Natural Resources Research and Education Center, AREEO, Sari, Iran

Email corresponding author: Ramesh.katam@famu.edu

Pistachio (*Pistacia vera* L.) is an economically important tree nut that commonly thrives in semi-arid and arid environments. *P. vera* is highly adaptable to various abiotic stresses, and its tolerance to drought and salinity stresses, makes it suitable for reforestation of arid and salinized zones. However, the mechanisms underlying the salinity tolerance of this plant are not well understood. The present study was aimed at physiological and molecular investigations to unravel the metabolic pathways associated with the salt tolerance mechanisms in UCB-1cultivar. Five one-year-old pistachio rootstocks were treated with four saline water regimes for 100 days. The rootstocks adopted Na+ exclusion strategy to resist the salinity stress. Total proteins were isolated from the roots and treated with different NaCl concentrations. The proteins were characterized using high throughput LC-MS/MS spectrometry searched against *the Citrus* database. Over 1600 protein IDs were detected, among which the comparative analysis revealed 245-high abundant and 190 low abundant proteins to three stress levels. The proteins associated with amino acid metabolism, cell wall organization, protein homeostasis, response to stress, signal transduction, TCA cycle, and vesicular trafficking were constantly overexpressed at all stress levels. At low and moderate stress levels, the chromatin and cytoskeleton organization lipid metabolism proteins were overexpressed, while at higher salt concentrations, they were unaffected. Transcription and translation processes were affected by all stress levels, as the proteins showed down-regulation in response to all stress levels. Transcription proteins were downregulated at low and moderate stress, while overexpressed at high salt stress treatment. Protein interaction network with all the orthologous proteins mapped to *Arabidopsis thaliana* and the clusters associated with these proteins revealed the cytosolic, carbohydrate, and amino acid metabolism are associated with salinity stress tolerance. The proteome data were validated with corresponding changes in transcripts.

Keywords: *Pistachio*, salt stress, shot gun proteomics, Protein-protein interaction.

Stoichiometric determination of ferritin levels in *Medicago truncatula* under drought stress and its relation to *Rhizobium* symbiosis using the Mass Western.

Carlos Pérez-Rízquez[1], Lisa Carolin Bilz[1], Stefanie Wienkoop[1]

[1]Plant-Microsymbiont Interaction Group. Department of Functional and Evolutionary Ecology. University of Vienna.

Email corresponding author: carlos.antonio.perez.rizquez@univie.ac.at

Nitrogen-fixing rhizobacteria symbiosis plays a role in drought stress tolerance in legumes. It has previously been shown that symbiosis triggers a symbiont induced stay green (SISG) phenotype along a delay in leaf senescence upon drought. This also enables plants to recover from desiccation more rapidly. In our previous publications on *Medicago truncatula,* we reported that ferritin levels were systemically enhanced upon symbiont interaction during drought stress, independently of the *Sinorhizobium* strain. Ferritins have been shown to be involved in leaf senescence as well as drought response. We also found them to be involved in nodule formation. Hence, ferritin, as Fe-storage and distribution protein, is therefore a key protein to keep the functioning of the symbiotic interaction and, especially, its formation. As a consequence, ferritins are not only stay green marker for enhanced drought stress tolerance but also symbio-proteins as they additionally regulate nodulation. Two major isoforms appeared to be involved in these regulations. We want to know their distribution and stoichiometry in leaves along different treatments and genotypes. Here, the absolute amount of ferritin isoforms was quantified in two *M. truncatula* genotypes: wildtype R108 and the HapMap accession HM022. Two groups of plants of each genotype were grown. One group was inoculated with *Sinorhizobium meliloti* and the second one grew in a rhizobia-free medium with mineral nitrogen fertilizer. Stoichiometry determination of ferritin isoforms was achieved using the Mass Western approach. Two cross-concatenated internal standards labeled with heavy isotopes (13C/15N) were designed, each one consisting of one ferritin specific mono-labeled peptide and one unspecific double labeled (absent in *M. truncatula* proteome) peptide. Non-target peptides were used as equalizer peptides (EP) for normalization between samples and standards. We will discuss the data with a critical analysis of the Mass Western approach.

Keywords: Mass Western, ferritin, drought stress, Symbiont Induced Stay-Green Phenotype, *Medicago truncatula*.

Acknowledgment: Work supported by the Austrian Science Fund (FWF) - Symbioprotein Ferritin (SymProFerr) [P 33930] and COST action 19116 - Trace metal metabolism in plants.

Cross-omics analysis uncovers key transcription factors involved in UVC-inducible ripening signaling in peach fruit.

Nasiopoulou Elpida[1], Skodra Christina[1], Michailidis Michail[1], Adamakis Ioannis[2], Samiotaki Martina[3], Tanou Georgia[4], Ganopoulos Ioannis[4], Bazakos Christos[4], Dalakouras Athanassios[5], Molassiotis Athanassios [1]

[1]Laboratory of Pomology, Department of Horticulture, Aristotle University of Thessaloniki, Thessaloniki 57001, Greece
[2]Department of Biology, National and Kapodistrian, University of Athens, 15784 Athens, Greece
[3]Proteomic Facility, B.S.R.C. "Alexander Fleming", Vari, Attica, Greece
[4]Joint Laboratory of Horticulture, ELGO-DIMITRA, 57001 Thessaloniki-Thermi, Greece
[5]ELGO-DIMITRA, 57001 Thessaloniki-Thermi, Greece

Email corresponding author: elpinasi@agro.auth.gr

Peach is a climacteric fruit with a high respiration rate and, consequently, after its harvest, it tends to lose rapid weight, which is an undesirable commercial characteristic. Ultraviolet-C (UV-C) radiation is used as a postharvest treatment to prolong the shelf life of fruit. However, this stressful process may also affect ripening and, consequently, water loss. To test this hypothesis, 'Luciana' peach fruit was harvested, exposed to UVC irradiation for 10 minutes and ripened at room temperature (20 ºC) for 8 days in the absence or presence of cold storage (7 days at 0 ºC). Treatment with UV-C impaired several anatomic features of peach peel. Exposure to UV-C radiation altered peel color indicators, and reduced the softening rate and the weight loss of fruit. Several primary and secondary metabolites were changed by UV-C in flesh and especially in peel samples. We identified various UV-C affected genes in both flesh and peel tissues following 8 hours of UV-C application. Particularly, UV-C specifically regulated the expression of a significant number of transcription factors, including ethylene response factor 1 (ERF1). To characterize the role ERF1 in UV-C ripening, we analysed its site-specific methylation and mutation status, and we also performed the ERF1gene *silencing by RNA interference* (*RNAi*) in peach fruit prior to UV-C exposure. Downregulation of ERF1 gene expression in peach peel tissue increased weight loss during ripening, decreased flesh firmness and affected the color of the fruit. Proteomic analysis following ERF silencing identified several ERF1-affected proteins. In conclusion, a relationship has been established between UV-C treatment and water loss, correlated to changes in ERF1 evaluated during the postharvest ripening of peach fruit.

Keywords: Ethylene response factor, peach fruit, peel tissue, ripening, *RNA interference*, UV-C radiation, water loss.

Acknowledgment: This work was supported by the project «On-tree ripening development for improvement of peach fruit quality» (Project code: ΔΜΡ1-0017915).

Insight on leaf proteome upon recovery to warm acclimation.

Norazreen Abd-Rahman[1], Giles N. Johnson[1]

[1]Department of Earth and Environmental Sciences, School of Natural Sciences, University of Manchester, United Kingdom

Email corresponding author: norazreen.bintiabdrahman@postgrad.manchester.ac.uk

Plants are almost always exposed to non-optimal temperatures at times throughout their life cycle. In this paper, we have performed a label-free proteomics approach to investigate the recovery responses of *Arabidopsis thaliana* (Col-0) of post warm-acclimation. Plants (Col-0) were grown for eight-weeks under control conditions (20 °C/ 18 °C) and acclimated to warm (30 °C/ 25 °C) for one week. Following acclimation, plants were returned to growth conditions for one-week recovery. In comparison to control plants, acclimation to warm ambient has increased total protein leaf content in recovery plants. Following proteomics analysis, also revealed that warm acclimation has induced changes in the proteome of recovery plants indicating that the underlying mechanisms are highly dynamic.

Keywords: warm temperature acclimation, photosynthesis, proteomics.

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Growth in Chernobyl affects biochemistry and physiology of aquatic plants: Implications for resistance to pathogens.

Shubhi Mishra[1], Dmitri Gudkov[2], Peter Baráth[3], Olha Lakhneko[1], Maksym Danchenko[1]

[1]Plant Science and Biodiversity Centre, Slovak Academy of Sciences, Nitra 95007, Slovakia
[2]Institute of Hydrobiology, National Academy of Sciences of Ukraine, Kyiv 04210, Ukraine
[3]Institute of Chemistry, Slovak Academy of Sciences, Bratislava 84538, Slovakia

Email corresponding author: shubhi.mishra@savba.sk

Plants compromise their growth and development to tolerate stress factors. Ionising radiation is a ubiquitous challenging factor that induces molecular and cellular changes in plants. It may cause water radiolysis, consequently generating excessive reactive oxygen species. In response, plants adapt by synthesising the defence proteins and metabolites, including antioxidants. Moreover, chronic irradiation may affect plant resistance to subsequent biotic stress. Our study focused on revealing biochemical mechanisms responsible for the reaction of chronically irradiated wild aquatic plants (common reed—*Phragmites australis*) challenged with pest or pathogen attack. Mature leaves collected from the contaminated (primary with radionuclides ^{137}Cs and ^{90}Sr) and reference lakes in the Chernobyl Exclusion Zone were used as experimental material. We performed protein profiling using ultrahigh-performance liquid chromatography and direct mass spectrometry quantification. Among 1340 quantified proteins, we revealed 174 differentially accumulated proteins between control and contaminated locations. Principal component analysis showed that sampling variables had a higher impact on proteome than contamination with radionuclides. Samples from contaminated lakes showed higher fungal infection in leaf-sheath assay performed in the laboratory. This experiment will be followed by testing the pest infestation on irradiated plants using a polyphagous aphid. Subsequent milestones include the measurement of major antioxidant enzymes and studying site-specific protein carbonylation using affinity enrichment and mass spectrometry. The outcomes of our research will bridge fundamental radiobiology and relevant management practices for contaminated lakes.

Keywords: ionising radiation, plant immunity, common reed, oxidative stress, protein carbonylation.

Acknowledgement: This study was supported by the projects APVV-20-0545 and VEGA 2/0106/22.

PLANT INTERACTIONS AND SIGNALING

Comparative proteomic analysis of *Arabidopsis* roots treated with auxin and humic substances reveals different biostimulatory modes.

Leonard Barnabas Ebinezer[1], Ilaria Battisti[1], Micaela Pivato[1,2], Laura Ravazzolo[1], Silvia Quaggiotti[1], Antonio Masi[1], Giorgio Arrigoni[2,3], Paolo Carletti[1]

[1]Department of Agronomy, Food, Natural Resources, Animals, and Environment, University of Padova, Viale dell'Università, 16, 35020 Padova, Italy
[2]Proteomics Center of Padova University, Padova, Italy.
[3]Department of Biomedical Sciences, University of Padova, Padova, Italy

Email corresponding author: antonio.masi@unipd.it

The beneficial effects of humic substances (HS) on plant growth have been well-known and are often described as a hormone like effect. While there is information regarding the impact of exogenous auxin on the proteome of plants, the biological processes stimulated by HS, especially at the proteomic level are not well understood to date. Therefore, in the present study, an iTRAQ-based quantitative proteomic analysis of *Arabidopsis* roots treated with auxin (IAA) and standard humic substances (SHS) obtained from the International Humic Substance Society (IHSS), was performed to highlight similarities and differences in the metabolic response to these biostimulants. *Arabidopsis* plants treated with SHS had significantly altered fresh weight of leaves and roots. Total protein content in leaves and roots was lower in IAA-treated plants, and reduced root protein content was also observed with SHS treatment compared to control plants. Functional categorization of 99 differentially abundant proteins (DAPs) in IAA treatment and 79 DAPs identified in response to SHS treatment indicated that the most represented categories were related to translation, post-translational modification and protein turnover, followed by energy production and metabolism and transport of carbohydrates, amino acids and secondary metabolites. The most altered biological processes were also related to response to stimulus and cellular and metabolic processes based on the GO categorization. Pathway analysis indicated that the metabolic and biosynthetic pathways of carboxylic acids, amino acids such as phenylalanine, tyrosine and tryptophan, and glutathione were significantly enriched in the IAA dataset. Ribosome-associated cellular processes and carbon fixation were significantly enriched among the DAPs in IAA and SHS treatments. There were several more specific biological processes that were induced distinctively either by IAA or SHS. Taken together, our comparative proteomic analysis has revealed the overlapping and distinctive biological processes induced by IAA and SHS in *Arabidopsis* roots.

Keywords: *Arabidopsis*, biostimulant, humic substances, indoleacetic acid, hormone.

Proteophenotyping: Possible role of a hypersensitive induced response protein in legume-rhizobium symbiosis establishment.

Stefanie Wienkoop[1], Mirko Desideri[1], Sebastian Schneider[1], Thomas Ott[2]

[1]Molecular Systems Biology Division, Laboratory of Plant-Microsymbiont Interaction, Department of Functional and Evolutionary Ecology, University of Vienna, 1030 Vienna, Austria
[2]Plant Cell Biology, Faculty of Biology, Albert-Ludwigs-University of Freiburg, Germany

Email corresponding author: Stefanie.wienkoop@univie.ac.at

Microbial endophytes are generally beneficial to plant growth and enhanced resistance to environmental perturbations such as drought and pathogen attacks. Hence, plants must be able to distinguish friends from enemies. This is especially important when controlling invasion of symbiotic rhizobium bacteria during nodule formation. Hypersensitive Induced Response Proteins (HIR) are membrane proteins that contain an SPFH (stomatins, pro-hibitins, flotillins and HflK/C) domain, belonging to a superfamily named PID (proliferation, ion and death). They were found to interact with several proteins involved in pathogens defense response such as leucine-rich repeat proteins. Previously, a HIR protein was identified as a major protein at the symbiosome membrane of *Lotus japonicus* root nodules a good indication that this HIR also plays a crucial role during mutualistic rhizobium-legume association. Here, we present proteomic and morphological data that show evidence for its involvement in nodule formation and possibly in switch off of nitrate regulated autoregulation of nodulation (AON).

Keywords: *Lotus japonicas,* hypersensitive induced response protein, symbiosomal membrane proteins, root nodule formation, Rhizobium symbiosis.

Downstream and upstream partners of AtMKK1 as part of the MAPK signaling pathways during salt stress response in *Arabidopsis*.

Tim Xing[1], Jeffrey Pepin[1], Frances Armas[1]

[1]Department of Biology and Institute of Biochemistry, Carleton University, Ottawa, Ontario, Canada.

E-mail corresponding author: tim.xing@carleton.ca

Mitogen-activated protein kinase cascades are one of the many systems that allow plants to survive and defend themselves against pathogens and other environmental stresses. Knockout of AtMKK1 enhanced salt tolerance. We found that AtMKK1 mutant plants tolerated elevated levels of salt during both germination and adulthood. Mass spectrometry analysis indicated that the level of the α subunit of mitochrondrial H^+-ATPase, mitochrondial NADH dehydrogenase and mitochrondrial formate dehydrogenase was increased in AtMKK1 knockout mutants upon high salinity stress. Our work also revealed AtMEKK1 and CRLK1as potential upstream protein partners of AtMKK1.

Keywords: AtMKK1, salt tolerance, CRLK1, AtMEKK1.

From *S*-sulfenylation to *S*-sulfinylation: redox switches in plants.

Jingjing Huang[1,2], Lindsy De Veirman[1,2], Zeya Chen[1,2], Patrick Willems[1,2,3,4], Didier Vertommen[5], Kate Carroll[6], Jing Yang[7], Frank Van Breusegem[1,2]

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052, Ghent, Belgium.
[2]VIB Center for Plant Systems Biology, VIB, 9052, Ghent, Belgium.
[3]Department of Biomolecular Medicine, Ghent University, 9000, Ghent, Belgium.
[4]VIB Center for Medical Biotechnology, VIB, 9000, Ghent, Belgium.
[5]de Duve Institute, Université Catholique de Louvain, Brussels, Belgium.
[6]UF Scripps Biomedical Research, Department of Chemistry, Jupiter, FL, 33458, USA.
[7]State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing, 102206, China.

Email corresponding author: Jingjing.huang@ugent.be

The harsher weather environments imposed by climate change causes increasing (oxidative) stress to plants, thereby triggering reactive oxygen species (ROS) production in plant cells. Hydrogen peroxide, a relative stable ROS, provokes reversible and irreversible oxidative post-translational modifications (OxiPTMs) on protein cysteine (Cys).The initial reaction of H_2O_2 with Cys thiols forms sulfenic acid (–SOH) that is intrinsically unstable and an intermediary en route to other OxiPTMs, such as the relatively more stable sulfinic ($–SO_2H$) and sulfonic ($–SO_3H$) acids, which are considered as overoxidized forms associated with protein damage and degradation. While $–SO_3H$ formation is irreversible, $–SO_2H$ can be recycled in an ATP-dependent manner by sulfiredoxin, a conserved eukaryotic antioxidant enzyme. In mammals, besides the well-known 2-cysteine peroxiredoxin substrates, more than 55 potential sulfiredoxin substrates have been identified using a $–SO_2H$ specific chemoproteomic workflow. However, in plants, so far only two sulfiredoxin substrates were reported: the chloroplast 2-Cys peroxiredoxin and the mitochondrial peroxiredoxin IIF. In order to expand the knowledge of plant sulfiredoxin substrates, our current work focuses on identifying the complete repertoire of sulfiredoxin substrates in plants using complementary interatomic and chemoproteomic approaches. Here, we will provide an overview of what we have learnt so far by re-evaluating plant sulfiredoxin and its substrates via proteomic and phenotypic approaches.

Keywords: Cysteine, oxidative post-translational modifications, sulfiredoxin, sulfinylation.

Acknowledgment: Work supported by Research Foundation-Flanders (FWO) Postdoctoral fellowship (no. 1227020N to J.H.).

Proteomics-based approach to identify novel players involved in autoregulation of nodulation in *Medicago truncatula*.

Sylwia Struk[1,2], Melissa Marreel[1,2], Annick De Keyser[1,2], and Sofie Goormachtig[1,2]

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium,
[2]VIB, Center for Plant Systems Biology, 9052 Ghent, Belgium

Email corresponding author: Sylwia.Struk@psb.ugent.be

Legumes establish a symbiotic relationship with nitrogen-fixing rhizobia via nodulation in which atmospheric nitrogen is converted into assimilable plant nutrients. As nodulation is energetically costly, the nodule number is tightly controlled through the process of systemic autoregulation of nodulation (AON). In *Medicago truncatula* the AON pathway is initiated in response to rhizobia (*S. meliloti*) by upregulating the expression of CLAVATA3/Embryo Surrounding Region (CLE) peptides, *MtCLE12* and *MtCLE13*. The Kelch repeat-containing F-box proteins TOO MUCH LOVE 1 and 2 (MtTML) are the key factors in the final stage of AON potentially functioning by ubiquitination and proteasome-mediated degradation of its targets. To identify the potential targets of MtTML1/2, as well as novel components of the AON pathway, we used shotgun proteomics in the *M. truncatula* composite plants with transgenic roots ectopically expressing *MtCLE13* or downregulating Mt*TML1/2*. Several proteins with different functions were detected as differentially accumulating in samples with overexpressed *MtCLE13* and downregulated *TML1/2* when compared to the control (*35S:GUS*). The involvement of identified proteins in nodulation was further validated by testing the expression profile of the corresponding genes in the *M. truncatula* roots at different time points after the inoculation with *S. meliloti*. The progress in this research will be discussed.

Keywords: nodulation, shotgun proteomics, *Medicago*.

Role of symbiotic rhizobacteria in drought resistance of tomato plants (*Lycopersicon esculentum*).

Anastasia Gurina[1], Nadezhda Frolova,[1] Elena Lukasheva[1], Alena Kuznetsova[1], Julia Shumilina[2], Katrina Alkhazhe[1], Tatiana Bilova[1,3], Anastasia Orlova[3], Svetlana Silinskaya[1],Maria Cherevatskaya[1], Alexander Shaposhnikov[4], Daria Syrova[4], Andrej Frolov[2], Andrej Belimov[4]

[1]Sant Petersburg State University, Universitetskaya emb., 7/9, St. Petersburg, Russia
[2]Leibniz Institute of Plant Biochemistry, Department of Bioorganic Chemistry, Weinberg 3, 06120, Halle/Saale, Germany
[3]K.A. Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, 35 Botanicheskaya str., Moscow, Russia
[4]All-Russian Research Institute of Agricultural Microbiology, Pushkin 8, hwy. Podbelsky, 3, St. Petersburg, Russia

Email corresponding author: st080515@student.spbu.ru

Symbiotic relationships with beneficial soil microorganisms are well-known for crop plants. For example, it was shown that inoculation of tomato plants with *Pseudomonas brassicacearum* Am3 strain, but not *P. brassicacearum* T8-1, is able to reduce the negative effects of drought on plant biomass. The observed effect could be related to the activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase in the bacteria. On the other hand, the ACC deaminase mutant T8-1 of Am3 strain demonstrated the absence of any protective effect on plants. As ACC is the precursor of ethylene, the activity of the enzyme and associated protective effect of the microorganisms are directly related to the hormonal status of plants. ACC uptake by bacteria stimulates it's outflow from plant roots, leading to decreased level of ethylene and hormonal inhibitory effect. However, despite the general understanding of the dynamics of hormone content in tissues, the fine molecular mechanisms behind the interaction of plant roots with rhizobial symbionts remain largely unknown. To address these mechanisms, tomato plants were grown in presence and absence of root colonizing bacteria under the conditions of drought and normal water supply. The content of hormones (abscisic acid, salicylic acid, auxins) and physiological parameters (leaf relative water content, photosystem II activity, stomatal conductivity and chlorophyll content in tissues) were measured in response to stress in the presence and absence of symbionts. The content of such biochemical stress markers as malondialdehyde and hydrogen peroxide was analyzed. A full-scale proteomic analysis was conducted in order to study the involvement of various enzymatic systems in the physiological response of plants to stress. Based on the data obtained, the proteome and physiological response of control and inoculated tomato plants to drought were characterized. The obtained proteomic data were also integrated with the obtained data of identified metabolites and their dynamics.

Keywords: *Lycopersicon esculentum*, PGPR, drought stress resistance, multiomics.

Acknowledgment: The work was supported by the project of the Ministry of Education and Science of Russia (Agreement No. 075-15-2922-320 dated 04/20/2022).

Chloroplast biogenesis: towards the role of localized translation in *Arabidopsis*.

Sara Pullara[1], Laura Dimnet[1], Lucas Moyet[1], Chloé Genevey[1], Samantha Moulin[1], Sabine Brugière[2], Alexandra Launay-Avon[3], Yohann Couté[2], Etienne Delannoy[3], Marcel Kuntz[1], Norbert Rolland[1]

[1]Laboratoire de Physiologie Cellulaire & Végétale, CNRS, INRAE, CEA, Université Grenoble Alpes, 17 Rue des martyrs, 38054 Grenoble, France
[2]Laboratoire Biologie à Grande Echelle, INSERM, CEA, Université Grenoble Alpes, 17 Rue des martyrs, 38054 Grenoble, France
[3]IPS2 Institut des Sciences des Plantes de Paris-Saclay, Plateforme Transcriptome POPS, Avenue des sciences, 91190 Gif sur Yvette, France

Email corresponding author: sara.pullara@cea.fr

Chloroplasts are major components of plant cells. Until recently, all nuclear-encoded proteins destined to chloroplast were believed to possess an *N*-terminal and cleavable chloroplast targeting peptide, and to engage the TOC/TIC machinery. However, recent studies revealed that alternative routes also exist and identified a series of nuclear-encoded proteins imported via such pathways. Recent proteomic studies, conducted by our team, identified a list of cyto-ribosomal subunits associated to chloroplasts, thus suggesting that localized translation might occur at the chloroplast surface. We were recently able to isolate plastid-associated cyto-ribosomes and to decipher their composition when compared to purified whole cell cyto-ribosomes. Interestingly, these plastid-associated cyto-ribosomes contain a few non cyto-ribosomal proteins which might participate to the control of localized translation at the chloroplast surface. These non cyto-ribosomal proteins were first fused to GFP to analyze their subcellular location. Then, we isolated *Arabidopsis* knock-out mutants affected by the expression of two of these specific proteins and initiated their phenotypical characterization. Finally, with the aim to identify the nature of the nuclear-encoded mRNAs that are translated by these chloroplast-associated cyto-ribosomes, the identification of mRNAs trapped within these plastid-associated cyto-ribosomes was performed. Surprisingly, very few of these mRNAs coded for chloroplast proteins.

Keywords: *Arabidopsis thaliana,* proteomics, localized translation, chloroplast, cytoribosomes.

Acknowledgment: Work supported by the Agence Nationale de la Recherche (ANR) Grants PolyGlot (PRC ANR-18-CE12-0021) and C-TRAP (PRC ANR-22-CE12-0012).

Identification of *Phytophthora* effectors targeting intracellular organelles in plants.

Siel Goethals[1,2], Laura Antuña Hörlein[1,2], Jonas De Backer[1,2], Monica Höfte[3], Inge De Clercq[1,2]

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9000 Ghent, Belgium
[2]VIB-UGent Center for Plant Systems Biology, 9000 Ghent, Belgium
[3]Department of Plants and Crops, Ghent University, 9000 Gent, Belgium

Email corresponding author: siel.goethals@psb.vib-ugent.be

Phytophthora species are notorious phytopathogens that reduce yield and quality of agricultural production and also affect our ecosystems. In order to obtain specific and sustainable solutions for plant protection, we need a better understanding of the molecular interactions between plants and pathogens. Intracellular organelles such as chloroplasts play a pivotal role in plant immunity and have been shown to remodel and relocate within the cell in response to various pathogen encounters. Increasing evidence suggests that pathogens secrete effector proteins, that directly interfere with organelle-initiated immune responses, but these organelle-targeting effectors, remain largely unidentified. Our project aims to identify novel organelle-targeting *Phytophthora* spp. effector proteins which will provide more insight in plant-pathogen interactions and the involvement of organelles in the plant's molecular response to biotic stresses.

Keywords: plant-pathogen interactions, effectors, intracellular organelles.

SYSTEMS BIOLOGY

A proteogenomics approach to unravel the quality of potato originating from contrasting terroirs.

Ifigeneia Mellidou[1], Anastasia Boutsika[1,2], Martina Samiotaki[3], Michail Michailidis[4], Theodoros Moysiadis[1], Christina Skodra[5], Aliki Xanthopoulou[1], Georgia Tanou[4], Maria Ganopoulou[6], George Stamatakis[3], Lefteris Angelis[6], Athanassios Molassiotis[5], Irini Nianiou-Obeidat[2], Christos Bazakos[1], Ioannis Ganopoulos[1*]

[1]Joint Laboratory of Horticulture, Institute of Plant Breeding and Genetic Resources, ELGO DIMITRA, Thessaloniki-Thermi 57001, Greece; [2]Laboratory of Genetics and Plant Breeding, School of Agriculture, Aristotle University, 54124 Thessaloniki, Greece; [3]Institute for Bioinnovation, Biomedical Sciences Research Center "Alexander Fleming", 16672, Vari, Greece; [4]Joint Laboratory of Horticulture, Institute of Soil and Water Resources, ELGO-DIMITRA, Thessaloniki-Thermi 57001, Greece; [5]Laboratory of Pomology, Department of Horticulture, Aristotle University of Thessaloniki, Thessaloniki-Thermi 57001, Greece; [6]School of Informatics, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

Email corresponding author: iganopoulos@elgo.gr

Potato is considered as the most important non-grain crop worldwide, whose quality can be remarkably influenced by genotype × environment interactions. Understanding how each distinct "terroir" can affect potato organoleptic properties is of outmost importance to develop new strategies towards the improvement of potato cultivation. As the dynamic interactions between plants and their surrounding environment are highly complicated, the identification of key molecular footprints associated with superior quality properties represents a great challenge. On the other hand, due to the limited depth of quantitative proteomics, our knowledge on gene/protein interacting networks remains largely untapped for the majority of plant cellular processes. In this study, we present one of the first proteogenomics approach to obtain the molecular portrait of potatoes (at harvest and at postharvest) which are widely cultivated in contrasting terroirs: one being the island of Naxos in the Aegean Sea (island region), producing the popular authenticated potatoes, and the other being a "control" site in the Northern Greece (continental region). Potatoes of the same genotype ("Spunta") but different origin have been previously found to exert astonishing differences in their organoleptic properties, evidencing that potato quality is shaped by terroir, including its microbiome. Transcriptome-based co-expression analysis of proteomic data revealed positive correlations for 53% between transcriptome and proteome datasets, with a 5% out of them being significant. By assessing transcript-protein pairs with values greater than 1 in at least one out of the four groups for both levels, a total of 1033 pairs were identified. Among them, there were a superoxide dismutase, a sucrose synthase, a starch synthase and two polyphenol oxidases. Conceptually, this innovative broad-scale quantitative and integrative work validated the expression of key gene markers by their protein abundance and could serve as a useful diagnostic tool to characterize and authenticate potatoes from different geographical origins.

Keywords: biomarkers, environmental footprint, proteomics, *Solanum tuberosum*, transcriptomics.

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A molecular study of Italian ryegrass grown on Martian regolith simulant.

Roberto Berni[1], Céline C Leclercq[1], Philippe Roux[2], Jean-Francois Hausman[1], Gea Guerriero[1], Jenny Renaut[1]

[1]Luxembourg Institute of Science and Technology (LIST), Environmental Research and Innovation (ERIN) Department, L-4940 Hautcharage, Luxembourg.
[2]Gembloux Agro-Bio Tech, TERRA Teaching and Research Centre, University of Liège, B-5030 Gembloux, Belgium.

Email corresponding author: jenny.renaut@list.lu

In the last decade, the exploration of deep space has become the objective of the national space programs of many countries. The International Space Exploration Coordination Group has set a roadmap whose long-range strategy envisions the expansion of human presence in the solar system to progress with exploration and knowledge and to accelerate innovation. Crewed missions to Mars could be envisaged by 2040. In this scenario, finding ways to use the local resources for the provision of food, construction materials, propellants, pharmaceuticals is needed. Plants are important resources for deep space manned missions because they produce phytochemicals of pharmaceutical relevance, are sources of food and provide oxygen which is crucial in bioregenerative life support systems. Growth analysis and plant biomass yield have been previously evaluated on Martian regolith simulants; however, molecular approaches employing gene expression analysis and proteomics are still missing. The present work aims at filling this gap by providing molecular data on a representative member of the Poaceae, *Lolium multiflorum* Lam., grown on potting soil and a Martian regolith simulant (MMS-1). The molecular data were complemented with optical microscopy of root/leaf tissues and physico-chemical analyses. The experimental plants grew for 2 weeks on regolith simulants andtheir leaves were bent downwards and chlorotic, their roots developed a lacunar aerenchyma and small brownish deposits containing Fe were observed. Gene expression analysis and proteomics revealed changes in transcripts related to the phenylpropanoid pathway, stress response, primary metabolism and proteins involved in translation and DNA methylation. Additionally, the growth of plants slightly but significantly modified the pH of the regolith simulants. The results here presented constitute a useful resource to get a comprehensive understanding of the major factors impacting the growth of plants on MMS-1.

Keywords: In situ resource utilization, *Lolium multiflorum*, Martian regolith simulants, Microscopy, Proteomics, qPCR.

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New insights into the stilbene biosynthetic pathway in mulberry (*Morus alba*) cell cultures via a proteomic approach.

María José Martínez-Esteso[1], Susana Sellés-Marchart[1,2], Ascensión Martínez-Márquez[1], Jone Etxeberría[3], Antonio Samper-Herrero[1], José Tomás Matus[3], Roque Bru-Martínez[4,5].

[1]Plant Proteomics and Functional Genomics Group, Department of Biochemistry and Molecular Biology, Soil Science and Agricultural Chemistry, Faculty of Science, University of Alicante, Alicante, Spain.
[2]Genomics and Proteomics Unit, SSTTI Universidad de Alicante, 03690 Alicante, Spain.
[3]Institute for Integrative Systems Biology (I2SysBio), Universitat de València-CSIC, Paterna, Spain.
[4]Plant Proteomics and Functional Genomics Group, Department of Biochemistry and Molecular Biology, Soil Science and Agricultural Chemistry, Faculty of Science and IMEM, University of Alicante, Alicante, Spain.
[5]Alicante Institute for Health and Biomedical Research (ISABIAL), Alicante, Spain.

Email corresponding author: Roque.Bru@ua.es

Morus alba cell cultures were used as a biotechnological productive system leading to the production of stilbenes via our established elicitation procedure in *Vitis vinifera* which uses a combination of cyclodextrins with methyl jasmonate. Stilbenes, polyphenolic-type plant specialized metabolites involved in the plant defence system, were already found in around twenty plant families including, among others, Vitaceae and Moraceae, and exhibit numerous pharmacological activities. We found that major stilbenes accumulated in the extracellular medium in mulberry were t-resveratrol (tR) and its hydroxylated form oxy-resveratrol (OR). Several other new metabolites, being tR and OR derivatives, were also identified. In the search for the particular proteins involved in secondary metabolism, we investigated the changes in the proteome of a mulberry cell suspension in response to a 72-hour elicitation treatment. The biosynthetic route leading to stilbenes is known to start with phenylalanine that is metabolized through the phenylpropanoid route up to the p-coumaroyl CoA synthesis, shared by other phenolics, including lignins and flavonoids. The final condensation between one p-coumaroyl-CoA and three malonyl-CoA molecules is catalysed by stilbene synthase to produce tR. The tR is a precursor for a range of stilbenes through different type of reactions such as glycosylation, hydroxylation, methylation, prenylation, isomerization and oligomerization, of which only a few have been biochemically and genetically characterized. In this study, elicitation treatment led to a strong stimulation of the enzymes involved in the shikimate pathway, phenylpropanoid, stilbenes and flavonoids biosynthesis. A correlation between protein and accumulated metabolites allowed us to gain insights into their biosynthetic pathways. The major aim of this research relies on the discovery of the genes and enzymes involved in the biosynthesis of the bioactive and high-value natural stilbenes in mulberry to bridge the still many knowledge gaps in their biosynthetic pathway.

Keywords: mulberry, stilbene, mass spectrometry, proteomics, label-free quantification.

Acknowledgment: This work was supported by Spanish Ministry of Science and Innovation, MCIN/AEI/ 10.13039/501100011033 and FEDER "Una manera de hacer Europa" grants BIO2017-82374-R and PID2020-113438RB-I00, and Valencian Conselleria d'Innovació, Universitats, Ciencia y Societat Digital grant CIAICO/2021/167.

Proteomics of lettuce seedlings (*Lactuca sativa* L.) highlighted the promotion of root organ morphogenesis and leaf primary metabolism induced by an innovative waste-derived biofertilizer.

Daniela Oliva[1], Amalia Piro[1], Dante Matteo Nisticò[1], Ilaria Lania[2], Maria Rita Basile[2], Giuseppe Chidichimo[2], Silvia Mazzuca[1]

[1]Laboratory of Plant Biology and Plant Proteomics (Lab.Bio.Pro.Ve.) – Department of Chemistry and Chemical Technologies Università della Calabria, 87036 Rende, Italy
[2]Physical Chemistry (CFINABEC) Laboratory – Department of Chemistry and Chemical Technologies Università della Calabria, 87036 Rende, Italy

Email corresponding author: silvia.mazzuca@unical.it

Information regarding physiological and molecular responses of plants to the treatment with new biofertilizers is limited. In this study, an innovative iron-based Fenton Composted Biofertilizer (FCB), obtained in 8 days starting from solid urban waste using the Fenton reaction, was tested pure or in combination with the Biochair (1:1), to evaluate the effects on the commonly used crop *Lactuca sativa* L. var. *longifolia*. The growth rate, leaves and roots biomass, chlorophyll concentration and total soluble proteins of seedlings treated with the 20 g/L FCB biofertilizer showed significant increase in comparison with control seedlings but not with those grown in the FCB/Biochair mixture. Leaf proteomic analysis revealed that the FCB induced the up-regulation of proteins belonging to photosynthesis machinery and carbohydrate metabolism and promoted the up-regulation of the water stress response. Root proteomics indicated that FCB strongly induced the organ morphogenesis and development; root cap development, lateral root formation, post-embryonic root morphogenesis were the main biological processes enriched by the treatment. Overall, our data suggest that the addition of FCB formulation to the base soils might ameliorate plant growth by inducing carbohydrates, primary metabolism, and the differentiation of a robust root system. The growing demand for sustainable alternatives to common fertilizers makes us assume that the innovative iron-based Fenton composted product could be largely used as an eco-friendly biofertilizer in crop production.

Keywords: root and leaf proteomics, biofertilizer, Fenton reaction, urban waste, lettuce.

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An ABC transporter b family member (VvABC-B) of grapevine is a trans-resveratrol transporter out of grapevine cells.

Martínez-Márquez A[1,4], Martins V[3,4], Sellés-Marchart S[1,2], Gerós H[3,4], Corchete P[5], Bru-Martínez R[1,6]

[1]Plant Proteomics and Functional Genomics Group, Department of Biochemistry and Molecular Biology, Soil Science and Agricultural Chemistry, Faculty of Science and IMEM, University of Alicante, Alicante, Spain.
[2]Research Technical Facility, Proteomics and Genomics Unit, University of Alicante, Alicante, Spain.
[3]Center for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), Quinta de Prados, Vila Real, Portugal.
[4]Research Group in Applied Plant Biology and Innovation in Agrofood-Agrobioplant, Department of Biology, School of Sciences, University of Minho, Braga, Portugal.
[5]Department of Plant Physiology, University of Salamanca, Salamanca, Spain.
[6]Alicante Institute for Health and Biomedicine Research (ISABIAL), Alicante, Spain.

Email corresponding author: asun.martinez@ua.es

Vitis cell cultures respond to pathogens and elicitors by synthesizing and extracellularly accumulating stilbenoid phytoalexins. Large amounts of trans-resveratrol (*t*-R) are produced when a cell culture is elicited with methylated cyclodextrins (MBCD), either alone or combined with methyl jasmonate. *t*-R transport to the extracellular medium, which represents the apoplastic space, would place this antifungal defense right in the battlefield to efficiently fight against pathogen attack. Yet despite their physiological relevance, these transport pathways are mostly unknown. Transcriptomic and proteomic experiments in elicited *Vitis* cell cultures, have shown an increase in the expression levels of MATE and ABC-type transporters, as well as glutathione-S-transferases (GST). A proteomic experiment on 72h-elicited grapevine cell cultures plasma membrane and tonoplast fractions was performed to explore the expression profiles of t-R biosynthetic proteins and other co-expressing proteins potentially involved in such a cell response. Among the proteins with differential abundance between elicited and non-elicited grapevine cell cultures we focused on a protein annotated as ABC-B transporter. Its encoding gene was cloned and stably expressed in both yeast and *Sylibum marianum* cell culture heterologous systems satisfactorily. We found that resveratrol transport occurs more intensively in transformed heterologous systems than in controls. Moreover, the functionality of the ABC-B transporter was analyzed by transient expression in *Vitis* cell cultures. In contrast to wild-type or GFP-expressing, ABCB- or GSTU10/ABCB-expressing cell suspensions showed a greater accumulation of extracellular *t*-R when an absorbent compound such as PVP or an elicitor such as MBCD were added in the culture medium at low concentration. The transient co-expression of both stilbene synthase and ABCB also showed a greater accumulation of *t*-R than wild-type or GFP-expressing cell suspensions. These results provide strong evidence of the role of this ABCB transporter in *t*-R trafficking to the extracellular medium in grapevine cells.

Keywords: Grapevine cell culture, proteomic analysis, resveratrol, extracelullar medium, ABC transporter.

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DEVELOPMENTAL PROTEOMICS

The role of the secretome in the early stages of somatic embryogenesis in *Pinus nigra*.

Miroslav Perniš[1], Terézia Salaj[1], Jana Bellová[2], Maksym Danchenko[1], Peter Baráth[2], Katarína Klubicová[1]

[1]Institute of Plant Genetics and Biotechnology, Plant Science and Biodiversity Center, Slovak Academy of Sciences, Akademická 2, P.O. Box 39A, 950 07 Nitra, Slovakia
[2]Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovakia

Email corresponding author: katarina.klubicova@savba.sk

Somatic embryogenesis is a suitable method for the rapid multiplication and preservation of valuable conifer trees. In addition, it represents an attractive model system to study the processes during embryo development. Extracellular proteins are essential regulators of somatic embryogenesis. Suspension cultures offer a convenient and non-destructive way to examine extracellular proteins secreted into a liquid cultivation medium. We used a proteomic approach to study the secretome related to the somatic embryogenesis of *Pinus nigra*. Considering differences caused by genotype, we analysed four cell lines with different regeneration capacities. Proteins acting on the cell wall polysaccharides, amylase and two basic secretory proteins were present in higher amounts in the medium from cell lines with high regeneration capacity. The medium from cell lines with low embryogenic capacity contained a higher concentration of extracellular nuclease and proteases, potentially acting in programmed cell death or nutrient recycling. In addition, we tested the peroxidase and amylase activity in the medium of several cell lines. We conclude that both tested enzyme activities are correlated with high embryogenic capacity and might be considered as potential markers of the early stages of somatic embryogenesis.

Keywords: Extracellular proteome, peroxidases, cell wall, amylase, black pine.

Can proteomics and transcriptomics elucidate trap leaf development in carnivorous sundews?

Rohan Arjun Kale, Martin Jopčík, Olha Lakhneko, Jana Libantová, Shubhi Mishra, Maksym Danchenko

Plant Science and Biodiversity Centre, Slovak Academy of Sciences, Nitra 95007, Slovakia

Email corresponding author: maksym.danchenko@savba.sk

One of the key features of carnivorous plants is the competency to trap and digest several types of prey utilising hydrolytic enzymes. This extraordinary capability provides vital nutrients while growing on deficient soils, such as marshes. Carnivorous plants evolved leaves with the special function of trapping, digesting, and absorbing the desired nutrients, complementing photosynthesis. Sundews are important group of carnivorous plant species, with their leaves modified into adhesive traps covered with glandular tentacles containing sticky secretion, filled with digestive enzymes. The development of these specialised leaves is poorly understood. Knowing the ontogenetic changes at the molecular level and correlating them with morphological changes would facilitate the deciphering of molecular mechanisms. Upon selection of individual developmental stages of trap leaves of *Drosera binata*, we will perform parallel RNA-Seq and proteomic analysis. RNA will be extracted by customised protocol and commercially sequenced, followed by bioinformatic analysis for relative quantification. Proteins will be extracted with detergent-containing buffer, digested with single-pot solid-phase-enhanced sample preparation protocol and quantitatively measured using liquid chromatography coupled mass spectrometry. Identifying hydrolytic enzymes regulated during trap development will create a list of candidate genes for deeper functional investigation. They will be a valuable genetic resource for exploring potential use in crop improvement, waste recycling, and medicine.

Keywords: carnivorous plants, *Drosera binata*, proteomics, transcriptomics, hydrolases.

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TECHNICAL/ METHODOLOGICAL ADVANCES IN PROTEOMIC ANALYSIS

Always in pursuit of time savings: from protein extraction to nanoLC-MS analysis for in-depth analysis of various tissue type.

Céline C. Leclercq[1], Roberto Berni[1], David M. Ribeiro[2], Olga Beine-Golovchuk[1], Quentin Enjalbert[3], André M. de Almeida[2], José A. M. Prates[4], Gea Guerriero[1], Jenny Renaut[1]

[1]LIST – Luxembourg Institute of Science and Technology (LIST), Environmental Research and Innovation Department, Bascharage, Luxembourg
[2]LEAF—Linking Landscape, Environment, Agriculture and Food Research Center, Associated Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal
[3]PreOmics GmBH, Martinsried, Germany
[4]CIISA - Centre for Interdisciplinary Research in Animal Health, Faculdade de Medicina Veterinária, Universidade de Lisboa, 1300-477 Lisboa, Portugal

Email corresponding author: celine.leclercq@list.lu

Proteomic sample processing has inherent challenges linked to sample preparation. We're still looking for solutions to save even more time in the lab. To implement a robust and reproducible standardized protein sample preparation, we used In-Stage tip (iST) technology applicable to all types of tissues prior to LC-MS analysis. iST kit coupled to BeatBox tissue homogenization platform allows significant time savings, sensitive and in-depth proteomic analysis, and a very easy-to-use workflow for everyone. A comparison of different sets of data from plants and animals will illustrate the benefits. We will compare in parallel protein extraction leaves and root tissues from a representative member of the Poaceae, *Lolium multiflorum* Lam. Plants were grown on potting soil vs. Martian regolith simulants (MMS-1) and homogenized with different instruments. Each protein sample was then digested and identified with NanoLC425 coupled to TripleTOF 6600+ mass spectrometry. Data were analysed by Progenesis QI for Proteomics. The following experiment compares muscle, hepatic and intestinal tissues of weaned male piglets randomly assigned to three dietary treatments containing seaweeds. Animal tissue samples were prepared with the iST workflow, and the peptides were separated in three fractions for in-depth analysis and compared to classical gel stacking protocol. Another case, we compare identification of a specific surexpressed protein from *Malus domestica* in *Nicotiana benthamania* in bioreactors, at different frequency available with the BeatBox to reach the optimal rate of extracted and digested proteins. This easy-to-use workflow combines novel homogenization techniques with BeatBox and iST kits for reproducible, faster and reliable sample preparation from cells and tissues.

Keywords: Proteomics, mass spectrometry, sample preparation, tissues.

Very short SDS-PAGE run coupled with in-gel tryptic digestion reduced the duration of mass spectrometry workflow unaffecting the protein identification quality in a seagrass species.

Daniela Oliva[1], Amalia Piro[1], Dante Matteo Nisticò[1], Faustino Scarcelli[1], Silvia Mazzuca[1]

[1]Laboratory of Plant Biology and Plant Proteomics (Lab.Bio.Pro.Ve.) – Department of Chemistry and Chemical Technologies Università della Calabria, 87036 Rende, Italy

Email corresponding author: silvia.mazzuca@unical.it

Seagrasses are recalcitrant to the common protein extraction methods due to high concentration of interfering molecules in their tissues. Therefore, time-consuming species-specific multistep workflows for free-label differential protein expression have been optimized to obtain high quality protein samples; these protocols were routinely coupled with the tryptic *in-gel* digestion as this method offers the advantage to remove any further contaminants (e.g., detergents, salts) during electrophoresis. The generated peptide samples can be, then, readily subjected to (LC/) ESI-MS analysis to obtain the peptide mass fingerprinting. The main drawback of *in-gel* digestion is that a single SDS-PAGE gel lane, corresponding to a biological or technical replicate, is excised, divided in several slices and *in-gel* digested following the common used protocol. By this procedure, sample is splitted in several independent digestion reactions and thus in multiple mass spectrometry analysis. In this way, the time lengthy and costly workflow is the main issue to face in the differential protein expression trials. In order to reduce the number of gel slices per sample, we shortened the duration of the electrophoresis from 1 hour to 20 min, using three acrylammide/bisacrylammide different percentages at same run conditions.

Keywords: *in-gel* digestion, SDS-PAGE, mass spectrometry, seagrasses.

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Protein carbonylation analysis of antibiotic-induced stress response in tobacco in vitro shoot culture.

Elena Andriūnaitė[1], Rytis Rugienius[1], Inga Tamošiūnė[1], Perttu Haimi[1], Jurgita Vinskienė[1], Danas Baniulis[1]

[1]Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry, Kaunas Str. 30, Babtai, 54333 Kaunas reg., Lithuania

Email corresponding author: elena.andriunaite@lammc.lt

Antibiotic timentin is used in plant in vitro tissue culture to eliminate microbial contamination following *Agrobacterium*-mediated plant transformation. Timentin has a relatively low cytotoxic effect on plant tissue culture. However, our previous studies have shown that antibiotic treatment induces an enduring growth-inhibiting effect in tobacco in vitro shoot culture that persists after tissue transfer to a medium without antibiotic. The effect was associated with an increase in $O_2^{\bullet-}$ concentration and oxidative lipid and protein injury. In this study, we assessed changes in oxidative protein modification in response to timentin treatment in tobacco (*Nicotiana tabacum* L.) in vitro shoot culture. Differential gel electrophoresis using carbonyl and amino group-reactive fluorescent dye protein derivatization revealed differential oxidative modification of 26 proteoforms of which the majority (22) had increased carbonylation content in timentin-treated tobacco shoot samples compared to the control. The enhanced carbonylation of four enzymes of the glycolytic pathway suggested a potential metabolic shift in glucose metabolism to the pentose phosphate pathway that could contribute to plant antioxidative response. In addition, an increase in carbonylation was detected for eight proteins related to the dark reaction of photosynthesis (including the Calvin cycle and photorespiratory carbon oxidation) and light-harvesting and energy transfer that could be related to the process of tissue senescence. Overall, the analysis suggested that timentin treatment-induced changes in protein carbonylation might be implicated in cellular redox balance regulation and accelerates the development of incipient senescence symptoms, and contribute to the shoot growth-suppressing effect of antibiotic treatment. The study demonstrates that the application of protein oxidative modification analysis is capable to provide important insights into the process of plant stress by revealing the non-translational aspect of stress response mechanism.

Keywords: in vitro stress response; oxidative stress; protein carbonylation; proteomics.

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Benchmarking commercially available trypsin proteases.

Petr Gintar[1,2], David Potěšil[1], Zbyněk Zdráhal[1,2]

[1]Mendel Centre for Plant Genomics and Proteomics, Central European Institute of Technology, Masaryk University, CZ-62500 Brno, Czech Republic
[2]Faculty of Science, Masaryk University, CZ-62500 Brno, Czech Republic

Email corresponding author: petr.gintar@ceitec.muni.cz

The bottom-up proteomics is a common approach used for protein characterisation. One of key steps of this approach is digestion during which proteins undergo proteolytic cleavage resulting in peptides which are subsequently analysed by various types of LC-MS/MS instrumentation. Up to date, different protocols using enzymatic digestion were introduced and applied in the proteomics research. The gold standard is still enzymatic digestion with trypsin serine protease. Trypsin is routinely used mainly for its high cleavage specificity, efficiency, broad temperature and pH optimum and reasonable price. Moreover, it provides peptides of optimal length for analysis by MS. Because of its high popularity and usage, different variants with modifications improving efficiency or preventing autolysis and chymotrypsin-like activity are now commercially available. Thus, researchers have an opportunity to choose among several types of trypsines mainly based on companies' information. In our work, we compared performance of commercially available modified trypsin enzymes and one trypsin/lysin-C mixture for digestion of 1 µg of plant sample. Samples were processed by single-pot solid-phase enhanced sample preparation (SP3) protocol. We tested several digestion parameters such as enzyme:substrate ratios, different incubation times with aim to select the best trypsin in terms of number of identified number of protein groups, proportion of missed cleavages and reproducibility. Our results will enable qualified selection of the most suitable trypsin for requested sample preparation in individual experimental set-ups.

Keywords: sample preparation, proteomics, mass spectrometry, SP3.

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One-minute alternative to reduction and alkylation of proteins in bottom-up proteomics.

Ondrej Šedo[1], Nela Pastyříková[1], Petr Gintar[1], Zbyněk Zdráhal[1]

[1]Proteomics Core Facility, Central European Institute of Technology (CEITEC), Masaryk University, Brno, Czechia

Email corresponding author: ondrej.sedo@ceitec.muni.cz

Reduction of disulfide bridges followed by alkylation of the liberated cysteines is a routine procedure in the proteomics analysis workflow. A rarely used alternative method achieves cleavage of the disulfide bridge by oxidation, which leads to the conversion of cystine to cysteic acid. This single reaction is performed using performic acid (prepared by incubating formic acid with hydrogen peroxide). The disadvantage of this method is the relatively long reaction time (3-4 hours) and the unwanted side reactions on other amino acid residues. In our work, a fast and simple method for the cleavage of disulfide bridges in proteins using performic acid under microwave irradiation in a common household microwave oven was developed. The conditions for protein treatment, with a mixture containing a high concentration of hydrogen peroxide and formic acid, were optimized. The final protocol, using a reaction time reduced to one minute, was tested on peptides, protein standards and plant protein mixtures by means of MALDI-MS/MS and LC-MS/MS. Reaction products at amino acid residues were determined and reflected by adequate protein modification settings in the MS/MS database search. In addition to speed, the main advantages of the proposed method consist in its ability to provide peptide signals from stable proteins inaccessible to a routine reduction and alkylation, and in the increased quality of the MS/MS data of peptides containing cysteine oxidized to cysteic acid.

Keywords: Proteomics, disulfide bridge, sample preparation, mass spectrometry.

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Proteomics approach for the detection and discovery of unknown allergens in almond (*Prunus dulcis*) pollen.

Antonio Samper-Herrero[1], Susana Sellés-Marchart[1,2], Ascensión Martínez-Márquez[1], María José Martínez-Esteso[1], María Ruano-Zaragoza[3], Víctor Soriano-Gomis[3], Javier Fernández[3], Roque Bru-Martínez[1,3]

[1]Plant Proteomics and Functional Genomics Group, Department of Biochemistry and Molecular Biology and Soil and Agricultural Chemistry, Faculty of Science, University of Alicante, Alicante, Spain.
[2]Research Technical Facility, Proteomics and Genomics Division, University of Alicante, Alicante, Spain.
[3]Allergy Group, Alicante Institute for Health and Biomedical Research (ISABIAL), Alicante, Spain

Email corresponding author: antonio.samper@ua.es

Although the main causing pollens of these respiratory allergies are known, there are others that are still unknown, either because the form of pollination does not involve the release of a large amount of pollen so being little studied, or because climate change has caused changes in the protein composition of pollen favouring the appearance of unknown allergens up to now. Proteomics is a key approach to detect new allergens or modifications of those already known. This knowledge would allow us to search for appropriate treatments that would prevent a large increase in this type of disease. A case that requires attention for these studies is almond pollen, since epidemiological studies have revealed allergic reactions in skin prick test of a population particularly exposed for either occupational or residential reasons or both. Going back to 1958, a scientific article is the first report in which a case of bronchial asthma due to the pollen of this species is verified. However, the presence of allergenic proteins in almond pollen has not been reported so far. In order to discover allergenic proteins in almond pollen a combination of proteomics tools has been used. After having confirmed the presence of allergenic proteins in almond pollen, the total protein extract was fractionated by anion-exchange chromatography, fractions resolved by 2-DE, IgE-immunoreactive spots detected by western blotting assays and identified by mass spectrometry. For the IgE detection assays by immunoblotting a pool of sera of the twelve sensitized individuals who were positive to almond pollen in skin prick test was used. Allergenic (Uniprot Allergen) and general (Uniprot Almond) protein databases were searched in order to identify already described and unknown allergenic proteins. Our investigations have allowed the identification of three new allergenic proteins (unpublished results).

Keywords: Proteomics tools, respiratory allergies, almond pollen, allergenic proteins, 2-DE.

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