Internal Report

Deliverable 3.3a:
Experimental setup for in-planta testing defined

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Abstract: Data integration in combination with modelling represent a strong tool for development of new hypotheses. Within the project, this approach is used to study the potato-PVY interaction system. Generated hypothesis have to be tested *in planta* using functional analysis tools. The exact experimental set up *in planta* depends of the hypothesis that we are testing. For functional analysis of potential key components, we are using virus-induced gene silencing (VIGS) as a fast screening system and short hairpin RNA-mediated (shRNA) gene silencing for further confirmation. Novel connections/paths can be tested using different approaches. We monitor predicted transcriptional changes after triggering a pathway artificially. On the other hand, potential protein-protein interactions are confirmed using yeast 2 hybrid (Y2H) and Co-Immunoprecipitation (Co-IP).
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Lead author:	Kristina Gruden
Co-authors:	Anna Coll, Živa Ramšak, Tjaša Stare, Špela Baebler
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Introduction

Within Task 3.3 from WP3 we are testing *in planta* the most interesting hypothesis generated on plant immune signalling from task 3.2. To that end we are using several methodologies that were previously developed at NIB or are being established throughout this project. For functional analysis of potential key components we are using virus-induced gene silencing (VIGS) as a fast screening system and short hairpin RNA-mediated (shRNA) gene silencing for further confirmation. Novel connections/paths can be tested using different approaches. We monitor predicted transcriptional changes after triggering a pathway artificially. On the other hand, potential protein-protein interactions are confirmed using yeast 2 hybrid (Y2H) and Co-Immunoprecipitation (Co-IP)

Materials and Methodology

- VIGS

At NIB we have recently established a virus induced gene silencing (VIGS) fast screening system for functional analysis of potato genes ***(1)***. The infectious clone of *Tobacco rattle virus* (TRV) carrying a partial sequence of gene of interest is introduced into *Solanum venturii* via agroinfiltration in order to silence the target gene. Later on, plants are infected with PVY^{N}-GFP ***(2)***. Virus spread is followed at different time points using confocal microscope.

- hpRNA silencing

To obtain stable transgenic plants with a target gene silenced we first produce a construct encoding a self-complementary hairpin RNA (hpRNA) that will recognize the gene of interest. The construct is introduce into *Agrobacteria thumefaciens* that will subsequently be used to infect potato plants. At NIB we have recently established a protocol to produce stable transgenic potato plants cv. Rywal and cv. Desiree.

- Analysis of transcriptional changes

To study transcriptional regulation we trigger a pathway artificially and monitor the predicted change. Specifically, we induce ethylene (ET), salycilic acid (SA) or jasmonic acid (JA) signalling pathways exposing potato plants to.ET, INA (analogue of SA) or methyl jasmonate (MeJA) respectively. We also inhibit the hormonal pathways by using 1-MCP (to inhibit ET signalling pathway), DIECA (for inhibition of JA). We follow predicted changes in gene transcription by qPCR using the protocols already established at NIB.

- Y2H

To confirm putative interactions between proteins, at NIB we optimised a protocol based in Y2H technology ***(3)***. We are using Matchmaker GAL4-based two-hybrid assay from Clontech. In this system, a target protein (bait) is expressed as a fusion to the Gal4 DNA-binding domain (DNA-BD), while the potential interacting partners (prey) proteins are expressed as fusions to the Gal4 activation domain. When the bait and the prey proteins interact, the DNA-BD and AD are brought into proximity to activate transcription of four independent reporter genes (AUR1-C, ADE2, HIS3, and MEL1).

Results

Experimental validation of network generated hypothesis of novel ET-SA crosstalk in potato
To confirm novel connections/paths we monitor predicted transcriptional changes after triggering a pathway artificially. From task 3.2 one of the most interesting findings is that the expression of the NPR1 gene should be modulated by the ethylene thus we tested the hypothesis by wetlab experiments.

Potato plant leaf samples (cv. Rywal) were sampled 24h after treatment ET, INA (SA analogue) or INA in combination with 1-MCP (ethylene pathway inhibitor). Gene expression of ACO4 (ethylene synthesis), PR1b (actuator in SA signaling) and NPR1 (signal transmitter in the SA pathway) were analysed. The results display a tight connection of SA-ET pathways, thus validation the connection between ET and NPR1 (Fig1).

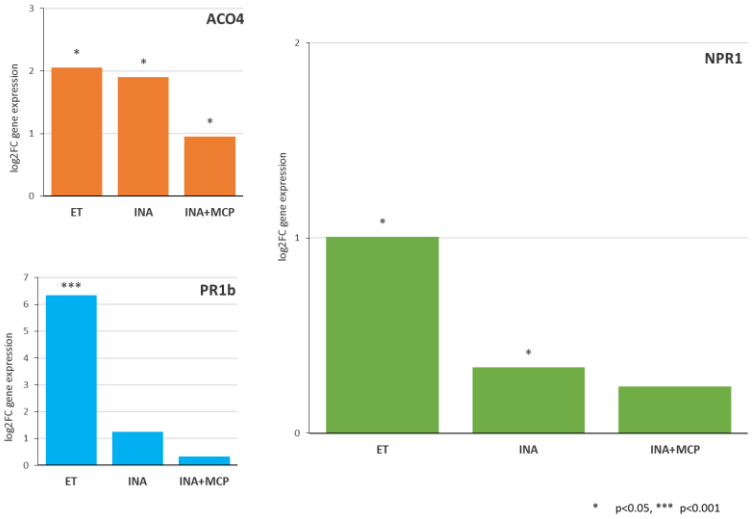

Fig 1. Analysis of transcriptional changes of NPR1

StSAPK a key component of potato defense response against PVY

A gene from the Osmotic Stress/ABA-activated Protein Kinase (SAPK) family was identified as a potential novel key components of PVY-potato interaction. To confirm the role of StSAPK in potato defence response we first silenced the gene using transient transformation. Gene expression analysis couldn't confirm the silencing of the gene, therefore we proceed with stable transformation using hpRNA silencing approach. Transgenic potato plants with StSAP knock-down were generated and now they we are growing them for further analysis (i.e. follow symptoms after virus inoculation and study the process of virus infection and propagation).
On the other hand, to get more insights into the regulation of this gene and to better understand the StSAPK/StPP2C (protein phosphatase 2C) signalling cascade we performed Y2H experiments using as a target proteins StSAPK and StPPC (Fig 2).

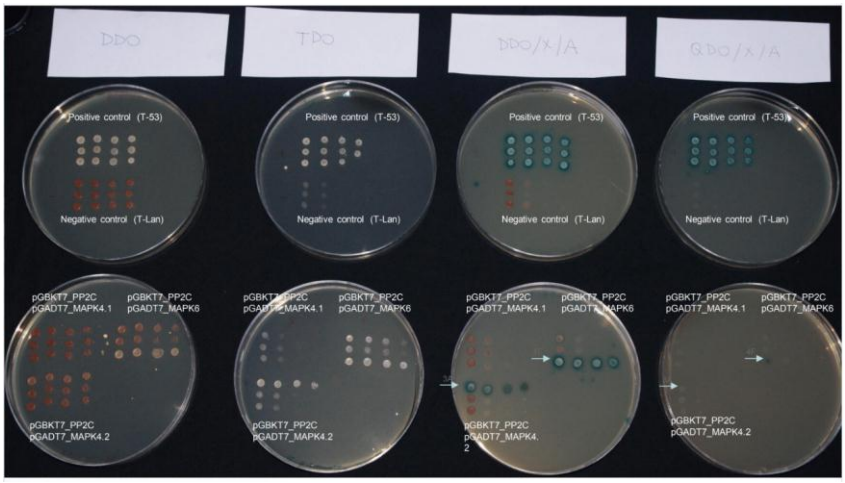

Fig 2. Yeast two-hybrid assays screening StPP2C interaction partners.

The results are shown in table1. Positive interactions are higlited in green.

Table 1. Results of Y2H experiments

	StMKP1	StPP2C	StAVR9
StSAPK	ν	ν	ν
StPK11	NA	∅	∅
StMAPK4.1		∅	∅
StMAPK4.2		ν	ν
StMAPK6		ν	ν

References

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2. M. Rupar, F. Florence, T. Michel, et al. (2015) Fluorescently Tagged Potato virus Y : A Versatile Tool for Functional Analysis of Plant-Virus Interactions, Molecular Plant-Microbe Interactions. 28, 739–750.
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