Applicant

Anna Coll Rius, National Institute of Biology, Department of Biotechnology and Systems Biology

Project

Identification and confirmation of protein-protein interactions involved in potato defence response against PVY

In the present project, 2 main objectives were proposed:

the first objective was **to screen genes involved in PVY-potato interaction against the potato cDNA library** available at the laboratory of Prof. Dr. Paul Birch (Bos et al., 2010) in order to isolate new putative partners for our target protein.

the second objective of the present project was to use **Co-IP technology to confirm potential interacting partners of some of our target protein kinases.**

Y2H Screening

We used The ProQuest™ Two-Hybrid System (Invitrogen) for detecting interactions between potato proteins *in vivo* in the yeast *Saccharomyces cerevisiae.* Two potato genes from Ethylen Response Famliy (ERF) that are involved in defence response signalling pathways were used as bait proteins to screen the **the potato cDNA library** available at the laboratory of Prof. Dr. Paul Birch (Bos et al., 2010). The Y2H screen was performed as described by McLellan et al. (2013)

- Prepare Bait vector, yeast transformation and check for auto-activation

Four potato genes, StERF.A1, StERF.C.6, StERF.C.6.1 and StSAPK, were cloned from pENTR-/D-TOPO (Invitrogen) to pDEST32 (Invitrogen) by means of LR reaction. *E.coli* transformants were analysed by Colony PCR and sequenced. After sequence confirmation, MaV203 yeast strain was transformed with the above mentioned destination vectors and grown in nutritional selection media to recover transformants.

Prior to the screening, bait plasmids were tested for self-activation. To that end, MaV203 cells were co-transformed with bait plasmids containing our target genes together with empty prey plasmid pDEST22 (Invitrogen). Yeast were plated on double-dropout media to recover double transformants. Four colonies from each transformation reaction were selected for further auto-activation assay. A strong and weak positive interaction control and a negative interaction control were also included. All 3 reporter genes (i.e. *lac*Z, *HIS*3 and URA3 auxotrophic markers) were tested. StERF.A1 showed weak autoactiavtion of lacZ reporter gene (see B-gal assay, Fig 1) and StERF.C.6 showed no autoactivation. On the other hand, StERF.C.6.1 and StSAPK presented strong autoactivation for His and lacZ reporter genes (Fig. 1), therefore they were discard as bait proteins for further screening experiments.

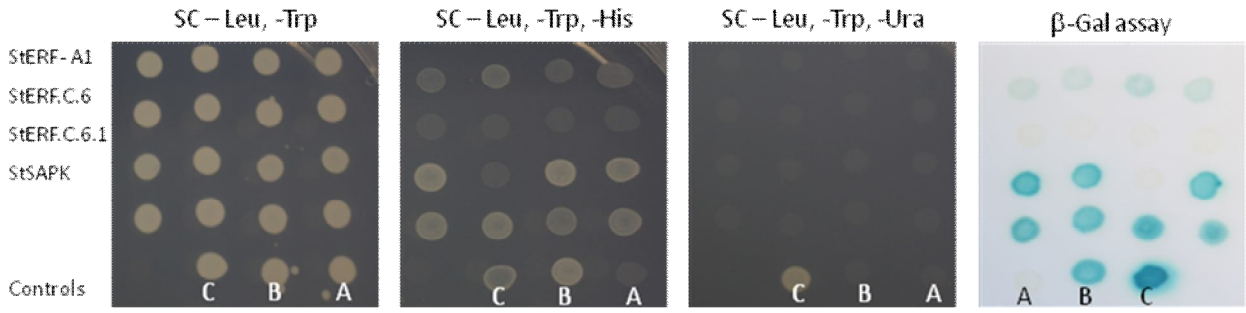

Fig. 1. StERF.A1, StERF.C.6, StERF.C.6.1 and StSAPK grew for 24h at 30C on different selective media. Double-dropout media (SC-Leu, Trp) was used for co-transformation selection. Autoactivation of bait proteins were examined by assessing growth on plates lacking histidine (SC-Leu,-Trp, -His) or uracil (SC-Leu,-Trp, -Ura) and performing X-Gal assay. A, B and C are a negative, weak positive and strong positive controls respectively.

- <u>Y2H screening</u>

Based on the results from auto-activation test, we proceed with large scale yeast transformation using StERF.A1 and StERF.C.6 as bait vectors.

Single transformants, that were previously obtained (see above "Prepare Bait vector, yeast transformation and check for auto-activation" section), were grown up and used to prepare competent yeast cells, which then were transformed with the potato Y2H "prey" library available at the host institute. After screening 6×10^6 yeast transformants for StERF.A1 and 1.6×10^6 yeast transformants for StERF.A1, 27 and 19 clones expressing potential interacting proteins with the respective baits were identified and further characterised.

- <u>Perform reporter assays and identify positive interactors</u>

Positive transformants were further characterised to confirm that the interaction induce all 3 reporter genes present in the system (His3, URA3 and LacZ). To that end, we assessed growing on plates lacking histidine or uracil and we performed x-Gal assay (Fig2).

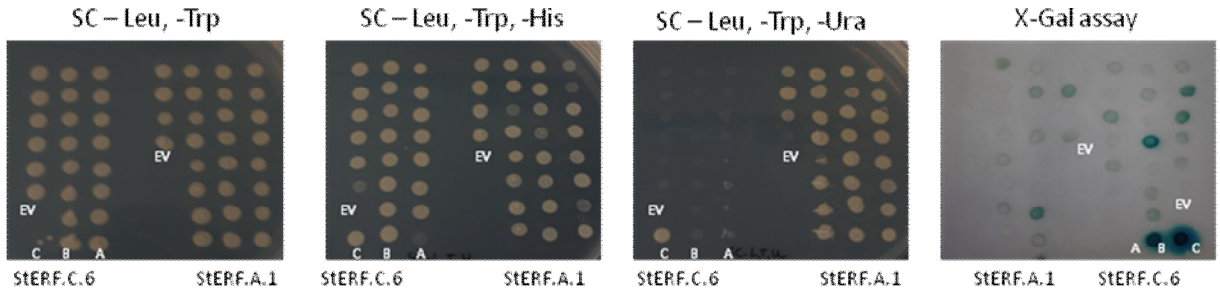

Fig. 2. Positive transformants from StERF.A1 and StERF.C.6 were grown at at 30C for 72h on different selective media. Double-dropout media (SC-Leu, Trp) was used for co-transformation selection. Induction of reporter genes was examined by assessing growth on plates lacking histidine (SC-Leu,-Trp, -His) or uracil (SC-Leu,-Trp, -Ura) and performing X-Gal assay. A, B and C are a negative, weak positive and strong positive controls respectively.

Only colonies that induced all 3 reporter genes were selected for plasmid isolation. Plasmids will be sequenced to identify interactors with StERF.A1 and StERF.C.6 respectively.

Co-IP

Two potato proteins StKinase (StK) and StPhosphates (StP) that were previously identified as potential interacting partners by pair-to-pair Y2H methodology in our lab were used in CoIP experiments in order to validate the interaction in planta. Briefly, *N. benthamiana* leaves were agroinflitrated with StK-GFP and StP_Myc. GFP-tagged fusion was immunoprecipitated using GFP-Trap-M magnetic beads. The resulting samples were separated by PAGE and Western blotted. Immunoprecipitated GFP fusions and co-immunoprecipitated c-Myc fusions were detected using appropriate antisera (Santa Cruz Biotechnology, UK) (Wang et al., 2015).

- Cloning, *agrobacteria* transformation and agroinfiltration

StK and StP were previously amplified from potato and cloned into pENTR-/D-TOPO (Invitrogen). StK entry clone was recombined with pB7WGF2 for N-terminal EGFP fusion; (Karimi, De Meyer, & Hilson, 2005) and StP entry clone was recombined with pGWB18 (for N-terminal c-Myc tagging).

Atumefaciens strain GV3101 was transformed by electroporation and four weeks old *Nicotiana benthamiana* were agroinfiltrated as described in McLellan et al (McLellan et al., 2013). Agrobacteria was cultured for 24 h in Luria Broth at 28°C supplemented with the appropriate antibiotics, spun at 4000 rpm and resuspended in 10 mM MgCl2:10 mM MES buffer with 200 µM Acetosyringone to OD600 = 0.8 before infiltration into *N. benthamiana* leaves.

- CoIP

Leaf disks were taken 48h after infiltration with constructs expressing either StK-GFP, StP-Myc or StK-GFP+StP-Myc. Plant material was ground in LN2 and proteins extracted with Tris-HCl buffer. Part of the protein extracts (*input*) were separated and directly loaded onto PAGE gel. The rest of the extracts were used to immunoprecipitate GFP-tagged StK fusions using GFP-Trap-M magnetic beads (Chromotek GmbH) (Wang et al., 2015).

Input and immunoprecipitated samples were loaded onto a 4-12% Bis-Tris NuPAGE Novex gel run with 1X MES running buffer for 1.5 h at 120 V (Invitrogen). Gels were blotted onto a nitrocellulose membrane for 1.5 h at 30 V and stained with ponceau solution to show loading and transfer (Fig 3). Membranes were blocked with 4%Milk 1xPBS buffer and subsequently incubated with appropriate dilutions according to the manufacturer instructions of either GFP antibody raised in rabbit or Anti MYC antibody raised in mouse. After washing the membrane with 1X PBST (0.2% tween 20) the secondary antibody was added at 1:10000 dilution of Anti-Rabbit IG HRP antibody or Anti-Mouse IG HRP antibody respectively. SuperSignal West Femto (Thermo Scientific) ECL detection was used according to the manufacturer's instructions (McLellan et al., 2013).

Although fig 3. shows that protein extraction loading and transfer were correct, only GFP was detected (data not shown). MYC fusion tag was not detected in any of the samples (no input neither immunprecipitated) revealing that StP-MYC was not successfully expressed in *N.benthamiana*. However, the methodology will be implemented at NIB and the experiment will be repeated.

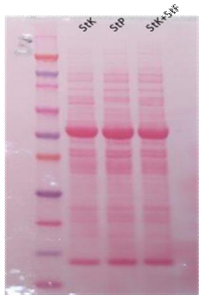

Fig3. Ponceau stain of input samples