Supporting Online Material for

Trojan Horse Strategy in *Agrobacterium* Transformation: Abusing MAPK Defense Signaling

Armin Djamei, Andrea Pitzschke, Hirofumi Nakagami, Iva Rajh, Heribert Hirt

DOI: 10.1126/science.1148110

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Supporting Online Material

Material and Methods

Yeast-two-hybrid assays
A yeast-two-hybrid screen was performed as described in (15), using MPK3 as DNA-binding bait, cloned in the vector pBTM116. Directed assays were performed according to (15) by cotransformation of yeast with VIP1 in pGAD425 and full-length MAPK cDNAs in pBTM116.

Plant material
A. thaliana Col-O plants were grown in single pots at 20°C at a 8h photoperiod, or on plates containing ½ MS medium (Duchefa), 1% sucrose and 0.8% agar (24°C, 16h photoperiod).

Transgenic expression in protoplasts
performed as described previously (16).

Plant protein extraction
Protein extracts of Arabidopsis protoplasts and seedlings were prepared as described in (17) and (18).

Treatment of seedlings with agrobacteria for kinase assay
12-day-old seedlings grown under sterile conditions were transferred for adaptation to ½ MS liquid medium over night. Following treatment with 10mM MgCl2 (mock) or a suspension of A. tumefaciens GV3101 grown to OD 0.8 in LB containing 25 mg/l rifampicin, washed and resuspended in 10mM MgCl2, the seedlings were shock-frozen in liquid nitrogen.

Expression and purification of GST fusion proteins
Escherichia coli strain BL-21 codon plus (Stratagene, La Jolla, CA) was transformed with pGEX4-T1 vector (Amersham-Pharmacia Biotech, UK) carrying the VIP1 coding sequence. Growth of bacteria and isolation of recombinant GST-fusion proteins were performed according to (19).

Kinase assay
MAPKs immunoprecipitated from protoplasts or leaves were incubated with recombinant purified GST, GST-VIP1 or MBP. Samples were processed according to (16).

**Coimmunoprecipitation and immunoblotting**

Protoplast extracts were processed according to (16) for coimmunoprecipitation with the antibodies polyclonal rabbit anti-MPK3, anti-MPK4, anti-MPK6, anti-HA and for immunoblotting with the antibodies polyclonal rabbit anti-MPK3, anti-MPK4, anti-MPK6, and monoclonal mouse anti-Myc and anti-HA.

**Plasmids and cloning**

Full-length VIP1 coding sequence, was amplified by PCR and cloned as NcoI – NotI fragment into pRT100 (20), pGex4T (Stratagene), pER8-YFP (21). The NotI site of VIP1_pRT100 and VIP1_pGreen0029 was further used to clone in-frame different C-terminal tags (hemagglutinin (HA), Myc or yellow fluorescent protein (YFP). The codon for the MAPK phosphorylation site (Ser79) of VIP1 was changed to alanine (S79A) or aspartic acid (S79D) by site-directed mutagenesis (Stratagene). ORFs of different MAPKs used were cloned as described by (22). For PR1::GUS, the intron-containing β-glucuronidase (GUS) gene was cloned into the binary vector pGreenII 0029 (23). A 833 bp region upstream of the PR1 (At2g14610) translational start was amplified by PCR from genomic Arabidopsis col-O DNA and subcloned 5´of the GUS gene. For Myr-VIP-YFP, the myristoylation/palmitoylation tag was generated by annealing of the oligos CATGGGATCCTGCTGCTCTCGAGCAACGTCTCCAGACTCTGGTAGGGGTGC and CATGGCACCCCTACCAGAGTCTGGAGACGTTGCTCGAGAGCAGCAGGATCC and cloned 5´ of VIP1 as an NcoI-NcoI fragment into pGreenII 0029 VIP1-YFP. In the resulting construct, the NcoI site was mutated to change the codon following the translational start methionin codon to a glycin codon using primers CCATTTACGAACGATAGCTATGGGATCCTGCTGCTCTCGAGCA and TGCTCGAGAGCAGCAGGATCCCATAGCTATCGTCTCGAGCA and verified by sequencing.

**Transgenic plants and localisation studies of VIP1-YFP, VIP1_A-YFP, VIP1_D-YFP**
Wild type *A. thaliana* ecotype Columbia plants were transformed with constructs VIP1-YFP, VIP1_A-YFP and VIP1_D-YFP (in vector pER8 containing an estradiol-inducible promoter (21)) by floral dipping (24). Leaves of 5-week-old plants of homozygous stable transgenic plants were floated for 12h on ½ MS medium supplemented with 40µM 17β-Estradiol (Calbiochem). For studying stress-induced relocalisation of VIP1, water (mock), 1µM flg22 (in water) or an agrobacterial solution of OD 0.9 (in water) were directly applied onto the leaf just prior to photography. Confocal images were taken with a Leica TCS SP camera (objective 63x1.32 – 0,6; oil immersion; excitation 460-490, emission 510-530). Data were processed using the Leica confocal software (Leica Microsystems, Heidelberg GMBH), Photoshop and Corel Draw. The microscopic data shown were reproduced in at least 6 independent lines and representative pictures are shown.

**GUS activity assay**

GUS activity of lysates from protoplasts and plant cell cultures was assayed as described (25) using the 1420-Multilable Counter Viktor3V (Perkin Elmer).

**Cell culture-based transient expression assay**

Transient Agrobacterium-mediated transformation of *A. thaliana* root cells was performed according to (26). Agrobacteria strains used were the virE3-deficient strain (27) with reporter vector 35S::GUSintron_pBin (28) and GV3101 incl. helper plasmid pSoup and the effector vectors VIP1, VIP1_A, VIP1_D as Myc fusions in pGreenII 0029 (23).
References


Figure S1

**Phosphorylation of GST-VIP1 by GST-MPK3**

Recombinant proteins were expressed in *E. coli* and purified. No phosphorylation was observed when the MAPK phosphorylation site of VIP1 was mutated (VIP1_A, VIP1_D). The upper band present in all GST-MPK3-containing samples in the autoradiography corresponds to autophosphorylation of GST-MPK3. Multiple bands observed for GST-VIP1,
GST-VIP1_A, GST-VIP1_D proteins correspond to degradation variants. No phosphorylation was detected in the absence of the kinase, excluding the possibility that VIP1-GST is autophosphorylating.

**Figure S2**

Subcellular localisation of VIP1_A-YFP after flg22 treatment

Confocal images of leaves from 5-week-old stable transgenic *A. thaliana* plants with estradiol-induced (40µM, 12h) VIP1_A-YFP expression. Leaves were treated with 1µM flg22 and pictures were taken at the time points indicated. Bar=20µM

**Figure S3**

Expression from CaMV35S promoter is not affected by VIP1, VIP1_A nor by VIP1_D

GUS activity in protoplasts 16h after transfection with 35S promoter::GUS construct alone (/) or in combination with a VIP1-HA, VIP1_A-HA or VIP1_D-HA construct. n=4, SD indicated. Expression of HA fusion proteins is visualized by immunoblotting.

**Figure S4**

The Trojan horse strategy in agrobacterial T-DNA transfer.

A PAMP-activated MAPK-cascade (MKK4/5 – MPK3) leads to the phosphorylation of VIP1. The phosphorylated VIP1 is transported in a karyopherin-dependent pathway through the nuclear pore complex (NPC) into the nucleus where it triggers the regulation of defence-related genes. Agrobacteria abuse the stress-induced nuclear import of VIP1 as a shuttle for their T-DNA complex by the interaction of VirE2 with VIP1.