TMV-Gate vectors: Gateway compatible tobacco mosaic virus based expression vectors for functional analysis of proteins

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SUPPLEMNTRAY INFORMATION

Supplementary Methods. Experimental procedures concerning construction of RfA cassettes, Entry clones, Agroinfection, electrophoresis analysis using Experion software and immunoblot analysis.

Supplementary Table S1. List of oligonucleotide primers used in this study.

Supplementary Figure S1. Nuclear localization of AtSAP18 expressed using TMV-Gate vector pMW391.

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SUPPLEMENTARY METHODS

Construction of RfA cassettes for TMV-Gate vectors

To assemble pTK249 encoding a 2xStrep-tag II-6xHis-Xpress epitope-linked RfA-cassette, pKR21 containing a resynthesised cassette encoding the 2x concatamer of Strep-tag II[1] was used as template in a PCR with the primers Strep-5'-PciNdePac and Strep-3'-Ase. The 165 bp amplicon was digested with PciI and AseI then ligated into pEXP1-Dest (Invitrogen) digested with PciI and NdeI, resulting in an in-frame fusion between 2xStrep-tag II and the 6xHis-Xpress epitope-linked RfA-cassette within pEXP1-Dest.

To create a destination cassette linked at its 3' end to the 2xStep-tag II-6xHis tag, 2xStrep-tag II was PCR amplified from pTK249 using the primers Strep-5'-NdeHind and Strep-3'-BamAge. The 180 bp amplicon was digested with HindIII and AgeI then ligated into pEXP2-Dest (Invitrogen) digested with the same enzymes, resulting in pMW394.

To create assemblies encoding the RfA-cassette linked to C-terminal YFP or CFP and 3x concatamers of the FLAG or HA tag, respectively, the RfA-cassette linked to C-terminal YFP or CFP was first amplified by PCR using pEarleyGate101 and pEarleyGate102[2], respectively, as templates and the primers Dest-PEG-5'-SnaMfePac and FP-HA-3'-SphSpe. The ~2.6 kb amplicons were digested with MfeI and SpeI then ligated into pLitmus38 (New England Biolabs) digested with the same restriction enzymes, resulting in plasmids pMW393 and pMW392 encoding RfA-YFP or RfA-CFP, respectively. Epitope tags encoding triple repeats of HA and FLAG epitopes were amplified using the primer sets HA-5'-SacBgl combined with HA-3'XbaSpe and FLAG-5'-SacBgl combined with FLAG-3'-XbaSpe, respectively, and pWY192 as template, a plasmid containing a resynthesized cassette encoding concatamers of HA and FLAG. The resultant 178 bp and 151 bp amplicons encoding 3xHA and 3xFLAG tags, respectively, were digested with SacI and SpeI then ligated into the same restriction sites of pMW392 and pMW393, respectively, resulting in a RfA-YFP-3xFLAG cassette designated as pMW397 and a RfA-CFP-3xHA cassette designated as pMW396 with pLitmus38 as the plasmid backbone in each case.

Construction of Gateway Entry clones

To construct a Gateway Entry clone encoding GusPlus, pCAMBIA 1305.1 (http://www.cambia.org/daisy/bioforge_gusplus/3703.html) was first digested with AfiII followed by treatment with the Klenow fragment of DNA polymerase to make blunt ends, and then digested with NcoI. The resultant 2113 bp fragment was ligated into pENTR11 (Invitrogen) digested with NcoI and EcoRV, resulting in pSW45. GusPlus coding sequence amplified from pCAMBIA1305.1 contains an intron from the castor bean catalase gene near its 5'end. To remove this intron from the GusPlus coding sequence, pSW45 was digested with NcoI and SpeI, then the linearized vector (4.17 kb) was ligated with a 32 bp piece of DNA, generated by annealing the primers GusPlus-sense and GusPlus-antisense, that restores the complete GusPlus ORF, resulting in pSK6.

To create a Gateway Entry clone encoding mGFP5, a thermotolerant mutant of GFP with fluorescence spectra optimized for long wavelength ultraviolet or blue light[3], pWY102[4] encoding mGFP5 was digested with BamHI and NotI then the mGFP5 fragment was ligated into pENTR3 (Invitrogen) digested likewise, resulting in pMW350. This plasmid enables in-frame fusions of GFP with the N-terminal epitope tags encoded by TMV-Gate vectors after LR clonase reaction.

To create a Gateway Entry clone encoding AtDMC1^{G138D} with a defect in the Walker A box of the conserved ATPase motif, pTK112 encoding the wild type AtDMC1 ORF in pENTR3 (Invitrogen) was used as template with the oligonucleotides AtDMC1-G138D-Sense and AtDMC1-G138D-Antisense in combination with the QuickChange Site-Directed Mutagenesis kit (Stratagene) following the directions of the manufacturer, resulting in the plasmid pTK135.

To link a nuclear localisation sequence to mCherry[5], the mCherry ORF was first amplified by PCR using the primers mCherry-5'-BamNde and mCherry-3'-AscPsp then digested with NdeI and AscI and ligated into pKR13 digested likewise, resulting in pMK6. pKR13 encodes a resynthesized cassette (National Research Council, Saskatoon, SK) for the seven amino acid NLS of the SV40 large T-antigen, shown to facilitate nuclear targeting in plants[6], linked to a 2x-concatamer of the c-myc epitope and unique restriction sites cloned into pDONR-D-TOPO

(Invitrogen). pMK6 thus encodes an in-frame fusion of the NLS_2xc-myc cassette with mCherry in a Gateway Entry vector. To enable C-terminal fusions to NLS_2xc-myc_mCherry the in-frame stop codon was eliminated by PCR using pMK6 as template in combination with the primers NLS-mCherry-5'-CACC-Bam and NLS-mCherry-3'-Asc and the amplicon cloned into pENTR-D-TOPO using the Directional TOPO Cloning kit (Invitrogen) and following the directions of the manufacturer, resulting in pSK212.

The predicted ORF of XopD was resynthesized by Bio Basic Inc. The ORF was then amplified by PCR using primers XopDrs_5'_attB1 and XopDrs_3'_Gly_attB2 to incorporate attB1 and attB2 Gateway cloning sites. The PCR product was cloned into pDONR-Zeo vector (Invitrogen,) using BP clonase II enzyme mix (Invitrogen), resulting in the construct pSU73.

The Gateway Entry clones pSW45, pSK6, pMW350, pTK135 and pSU73 were linearized by digesting with PvuI, which uniquely cleaves the kanamycin resistance gene. The linearized fragments were gel-purified, and recombined with Destination vectors pSK101-106, pTK251, pMW388, pMW390-391, or pMW399 (Table 1) using LR clonase II enzyme mix (Invitrogen,).

Agroinfection

Transformed *A. tumefaciens* GV3101::pMP90 cells grown overnight at 30^0 C in Luria-Bertani medium supplemented with appropriate antibiotics were diluted 1:10 in the same medium supplemented with 20 µM acetosyringone and grown at 30^0C for another 5-6 hrs to an OD_{600} of approximately 1.0. Cells were harvested by centrifugation and resuspended in Agro-induction medium (10 mM MES (pH 5.7), 10 mM $MgCl_2$ and 100 µM acetosyringone) at an OD_{600} of 1.0. These cells were incubated at room temperature for ~12 h before infiltration into the abaxial surface of *N. benthamiana* leaves using a 1 mL syringe without needle.

Quantification of proteins by Experion automated electrophoresis analysis

The Pro260 Ladder contains nine proteins ranging from 10-260 kilodaltons (kDa) as well as a 1.2 kDa lower marker. A standard curve of migration time versus protein size (in kDa) for the nine proteins in the Pro260 sizing ladder was constructed. The protein peaks in each sample well are

bracketed by the 1.2 kDa lower marker and the 260 kDa upper marker. These markers were used to normalize the protein migration times in the sample wells with the Pro260 Ladder. The Experion software uses the normalized migration times with the standard curve to calculate the sizes of all the proteins detected in each sample well. Concentration of each protein in each sample well was estimated by using the peak correlation area of the 260 kDa upper marker.

Immunoblot analysis

Proteins (~20 µg) were separated on 10% SDS–PAGE gels and transferred onto PVDF membrane by electroblotting. Epitope-tagged proteins were detected by sequential incubation with commercial antiserum raised against HA (Invitrogen; 1:2500 dilution), FLAG (GenScript; 1:5000 dilution), c-myc (Millipore; 1:4000 dilution), Strep-tag II (Novagen; 1:4000 dilution) or 6xHis (Invitrogen; 1:5000 dilution) followed by peroxidase conjugated anti-rabbit IgG (Santa Cruz Biotechnology) at a dilution of 1:5000 and chemiluminescent detection using SuperSignal West Pico Substrate (Thermo Scientific**).**

SUPPLEMENTARY REFERENCES

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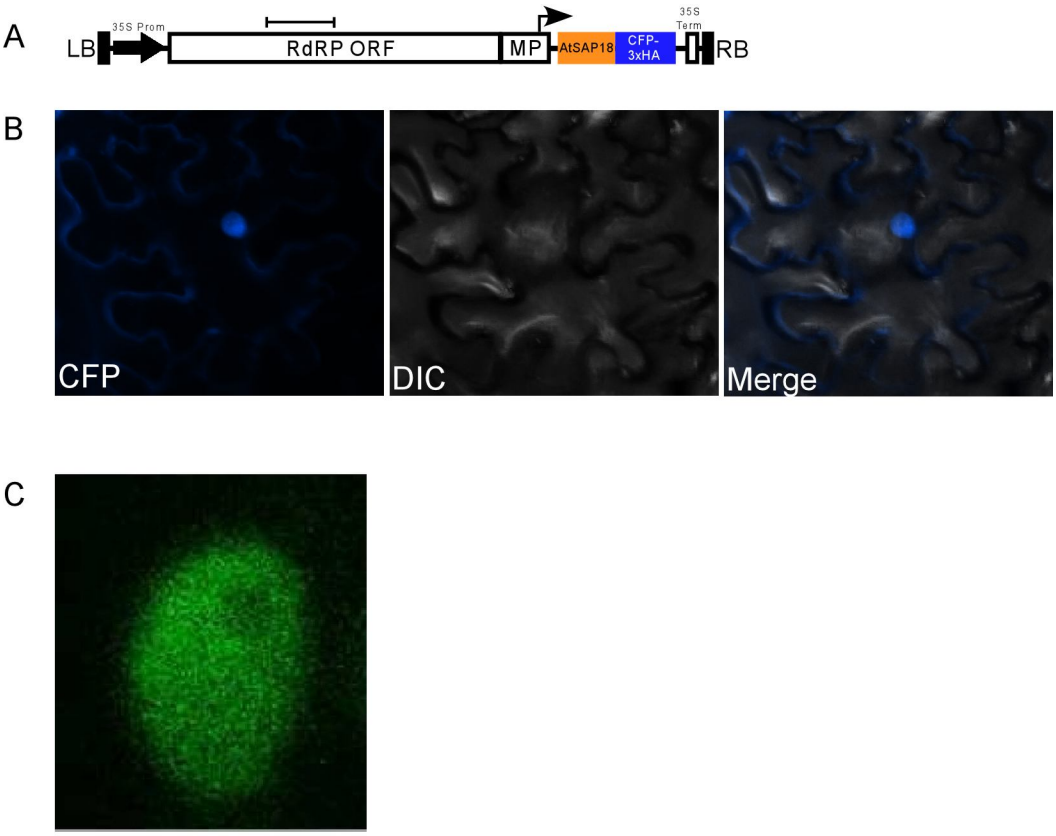
Supplementary Table S1. List of oligonucleotide primers used in this study

Primer name	Sequence (5'-3')
DEST--5'-SnaMfePac	TACGTACAATTGTTAATTAACGGTATCGATAAGCTTGATATCACAAG
pEG201-5'-BspPac	GATATCATGACTTTAATTAACATGTACCCATACGATGTTCCAG
pEG201-5'-BspPac+2xHA	GATATCATGACTTTAATTAACATGTATCCTTATGATGTTCCTGATTAT GCTGGTTATCCTTATGATGTTCCTGATTATGCTGGATCTTACCCATAC GATGTTCCAGATTACG
pEG202-5'-BspPac	GATATCATGACTTTAATTAACATGGACTACAAAGACGATGACG
pEG202-5'-BspPac+2xFLAG	GATATCATGACTTTAATTAACATGGATTATAAGGATGATGATGATAA GGGAGATTATAAGGATGATGATGATAAGGGAGACTACAAAGACGAT GACGACA
pEG203-5'-BspPac	GATATCATGACTTTAATTAACATGGAACAGAAACTGATCTCTG
pEG203-5'-BspPac+2xMyc	GATATCATGACTTTAATTAACATGGAACAAAAGCTCATTTCTGAAGA GGATCTTAATGGTGAGCAGAAATTGATTTCAGAGGAGGATTTGAAT GGAGAACAGAAACTGATCTCTGAAGAAG
DEST-3'AvrPsm	TACGTAGGGCCCCCTAGGCACCACTTTGTACAAG
DEST-3'-SphPspAvr	GTTAACGCATGCGAGCTCGGGCCCCCTAGGTCACTGCAGGAATTCGA TATCACCAC
STrEP-5'PciNdePac	GATATCACATGTCATATGTTAATTAACATGGCTGGAAGTACTGGAGC
EXP1-DEST-3'PspAvr	GATATCGGGCCCTAGGATCGAACCACTTTGTACAAGAAAG
STrEP-3'Ase	GATATCATTAATAGGCCTAGATCCAGCTCCC
STrEP-5'-NdeHind	TACGTACATATGGCTAAGCTTGAAGGACTTGAAGTTCTTTTTCAAGG TCCTGGATCTGGAGCTTCTTGGTCTCATCCTC
STrEP-3'-BamAge	GATATCGGATCCACCGGTAGATCCAGCTCCCTTTTCG
EXP2-DEST-5'-XhoSnaPac	GATATCCTCGAGTACGTATTAATTAACAAGAAGGAATTATCAACAA GTTTGT
EXP2-DEST-3'SacPspAvr	GATATCGAGCTCGGGCCCCCTAGGTCATCAATGGTGATGGTGATGAT G
DEST-5'-SnaMfePac	TACGTACAATTGTTAATTAACGGTATCGATAAGCTTGATATCACAAG
FP-HA_3'_SphSpe	TACGTAGCATGCACTAGTTCATTAAGCGTAATCTGGAACATC
HA-5'-SacBgl	TACGTAGAGCTCAAAGATCTGGAGGTCATAGAGGAGGTGGAAGAAT TT
HA-3'-XbaSpe	TACGTATCTAGAACTAGTCATCAAGATCCTCCATGAGCAGCATAAT
FLAG-5'SacBgl	TACGTAGAGCTCAAAGATCTGGAGGAAGCGCTGGAGATTATAAG

FLAG-3'-XbaSpe	TACGTATCTAGAACTAGTCATCAAGATCCAGTACTTCCCTTATCAT
XopDrs_5'_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTGGTACCAAAACAATG GAGTATATCC
XopD_3'_Gly_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGAACCTCCACCAAAC TTTTTCCACCACTTAGATTTC
AtDMC1-G138D-Sense	CACAGAGGCTTTTGGGGAATTTAGGTCTGATAAAACCCAATTAGCAC ATACCCTTTGTG
AtDMC1-G138D-Antisense	CACAAAGGGTATGTGCTAATTGGGTTTTATCAGACCTAAATTCCCCA AAAGCCTCTGTG
mCherry_5'_BamNde	GATATCGGATCCAAACATATGGTGAGCAAGGGCGAG
mCherry_3'_AscPsp	GATATCGGCGCGCCGGGCCCAAGCTTCGAATTCTTACTTGTAC
NLS-mCherry-5'-CACC-Bam	CACCGGATCCATGGCTCCAAAGAAGAAG
NLS-mCherry-3'-Asc	AAGCTTACTTCCACCTCCTCCAGATCCTCCACCTCCCTTGTACAGCTC GTCCATG
GUSplus-sense	CATGGTAGATCTGAGGAACCGACGAA
GUSplus-antisense	CTAGTTCGTCGGTTCCTCAGATCTAC



Supplementary Figure S1: Nuclear localization of AtSAP18 fused to CFP and expressed using TMV-Gate vector pMW391

A. Schematic diagram showing the construct used for analysis of subcellular localization of AtSAP18. The ORF of AtSAP18 was linked in-frame with the coding sequence of CFP-3xHA in pMW391. LB, T-DNA left border; RB, T-DNA right border; 35S Prom, CaMV 35S promoter; 35S term, CaMV 35S terminator; RdRP, RNA-dependent RNA polymerase; MP, movement protein; bent arrow, coat protein promoter; CFP, cyan fluorescent protein; HA, hemagglutinin.

B. Epifluorescent microscopy images showing nuclear localization of AtSAP18. AtSAP18 linked to CFP-3xHA expressed in cells of *N. benthamiana* leaves infiltrated with *A. tumefaciens* carrying the constructs described in (A) and imaged 3 DPI. Images were taken using CFP or DIC (differential interference contrast) filter sets as indicated.

C. Nucleoplasmic localization of AtSAP18 as demonstrated in the Arabidopsis nucleolar protein database (AtNoPDB; http://bioinf.scri.sari.ac.uk/cgi-bin/atnopdb/get-all-data?type=more&value=At2g45640)[7] by expressing its full-length cDNA–green fluorescent protein (GFP) fusion in *Arabidopsis* culture cells.