New Phytologist Supporting Information

Article title: **Chloroplast redox state changes mark cell-to-cell signaling in the hypersensitive response**

Authors: **Tjaša Lukan, Anže Županič, Tjaša Mahkovec Povalej, Jacob O. Brunkard, Mirjam Kmetič, Mojca Juteršek, Špela Baebler, Kristina Gruden**

Article acceptance date: 26th July 2022

Supporting Information Method S1: Stable Transformation

In the morning on the first day, one bacterial colony was cultured in 3 ml of YEB medium with selection; rifampicin (final concentration 20 µg/ml) and kanamycin (final concentration 50 µg/ml) and grown under standard conditions for agrobacteria. In the afternoon, 1000 µl of the morning bacteria culture was inoculated in 3 ml of YEB medium with selection and grown overnight.

In the morning on the second day, different volumes (between 200 and 1000 µl) of overnight bacteria culture were inoculated, each in 50 ml of YEB medium with selection. In the afternoon, different volumes (between 5 and 50 µl) of the morning bacteria culture in the exponential growth phase were inoculated, each in 50 ml of YEB medium with selection. Bacteria culture was grown overnight.

In the morning on the third day, overnight bacteria culture (OD_{600} = 0.4 - 0.7) was centrifuged for 10 min at 2500 rpm. Pellet was resuspended in 1300 µl of YEB medium without selection. Bacteria culture was spread on 10 plates with MS 30 medium (100 µl per plate). One plate with MS 30 medium with no bacteria was used as a control. On freshly harvested leaves and internodes (without the buds) several notches (two to four) were made with a sterile scalpel under sterile conditions. Leaves were placed with adaxial surface on the MS 30 medium. The plates with leaves and internodes were incubated at room temperature in the dark for two days. The plates were not taped.

On the fifth day, the explants were transferred onto MCI medium with the selection; cefotaxime (final concentration 500 mg/l) and hygromycin (final concentration 20 mg/l). The plates were taped with parafilm and incubated in a growth chamber for 10 days. Three plates with MCI medium were used as controls: the first plate was non-transformed positive control with MCI medium without hygromycin and without bacteria, the second plate was a positive control with MCI medium without hygromycin and bacteria, and the third plate was a negative control with MCI medium with hygromycin and without bacteria.

On the fifteenth day, the explants were transferred onto GR2 medium with selection. Three plates with GR2 medium were used as controls (described above). Explants were transferred onto fresh GR2 medium every two weeks. This procedure was repeated until sufficent number of transformants (shoots) were harvested on MS 15 medium with selection.

Media preparations:

For **YEB medium**, 5 g of beef extract, 1 g of yeast extract, 5 g of peptone, 5 g of sucrose and 0.5 g of MgSO4x7H2O were added in 1 l of bidistilled water, dissolved, calibrated to pH 7.2 and autoclaved.

For **MS 30 medium**, 5 g of Murashige and Skoog Basal Medium with vitamins and 30 g of sucrose were added in 1 l of bidistilled water, dissolved and calibrated to pH 5.8. Next, eight grams of agar were added to the solution and autoclaved.

For **MS 15 medium**, 5 g of Murashige and Skoog Basal Medium with vitamins and 15 g of sucrose were added in 1 l of bidistilled water, dissolved and calibrated to pH 5.8. Next, nine grams of agar were added to the solution and autoclaved.

For **MCI medium**, 5 g of Murashige and Skoog Basal Medium with vitamins and 16 g of glucose were added in 1 l of bidistilled water, dissolved and calibrated to pH 5.8. Next, eight grams of agar were added to the solution and autoclaved. Sterile 1-naphthaleneacetic acid (final concentration 5 mg/l), 6-benzylaminopurine (final concentration 0.1 mg/l), cefotaxim (final concentration 500 mg/l) and hygromycin (final concentration 20 mg/l) were added in sterile MCI media.

For **GR2 medium**, 5 g of Murashige and Skoog Basal Medium with vitamins and 16 g of glucose were added in 1 l of bidistilled water, dissolved and calibrated to pH 5.8. Next, eight grams of agar were added to the solution and autoclaved. Sterile gibberellic acid 3 (final concentration 0.02 mg/l), 1-naphthaleneacetic acid (final concentration 0.02 mg/l), trans-zeatine riboside (final concentration 2 mg/l), cefotaxim (final concentration 500 mg/l) and hygromycin (final concentration 20 mg/l) were added in sterile GR2 media.

Supporting Information Method S2: Construction of PVY-N605(123)-GFP infectious clone

PVY-N605(123)-GFP was constructed by inserting GFP coding sequence between the coding sequences for viral proteins NIb and CP in PVY-N605(123) infectious clone (Bukovinszki *et al.*, 2007) using a similar design as Rupar *et al.* 2015, allowing the GFP reporter to be excised from the polyprotein following translation.

GFP coding sequence was amplified from plasmid p2GWF7 (Karimi *et al.*, 2002) using Phusion® polymerase (New England BioLabs) with the following 25 µl reaction mixture and cycling conditions:

Component	Volume [µl]	Final concentration/amount
5X HF Buffer	5	1x
10 mM dNTP	0.5	200 µM
10 µM GFPF40	1.25	500 nM
10 µM GFPR40	1.25	500 nM
100 % DMSO	0.75	3 %
2U/µl Phusion	0.25	0.5 U
p2GWF7	1	10 ng
H2O	15	/

Temperature	Time	Step
98 °C	2 min	hold
98 °C 72 °C	8 s 40 s	35 cycles
72 °C	10 min	hold

In the GFPF40 and GFPR40 primers we included overhangs enabling addition of PVY-N605(123) annealing sequence and protease recognition site to the GFP sequence, making the amplicon serve as a megaprimer for restriction-free insertion with mutagenesis. After amplification, 1 µl of PCR reaction was examined with agarose gel electrophoresis. Since we detected only one band of correct size, the PCR reaction was used directly for the mutagenesis reaction, designed using the RF-Cloning online tool (Bond & Naus, 2012) and carried out using Phusion polymerase with 1:20 molar ratio of PVYN605(123) plasmid to GFP amplicon (megaprimer):

Component	Volume [µl]	Final concentration/amount
5X HF Buffer	4	1x
10 mM dNTP	0.4	200 µM
PVY-N605(123)	1	150 ng
GFP megaprimer	1	200 ng
2 U/µl Phusion	0.2	0.4 U
H2O	13.4	/

Temperature	Time	Step
98 °C	1 min	hold
98 °C 72 °C (decreased by 1 °C every cycle)	8 s 20 s	5 cycles
72 °C	7.5 min	
98 °C 68 °C 72 °C	8 s 20 s 7.5 min	13 cycles
72 °C	10 min	hold

To the reaction mix after amplification, 2 µl of *Dpn*I enzyme (Agilent) were added and reaction incubated for 2h at 37 °C. 2 µl of mutagenesis mixture were transformed into *E. coli* XL10-Gold Ultracompetent Cells (Agilent) using a 45 µl cell aliquote supplemented with 2 µl of β-mercaptoethanol and following the standard heat-shock transformation protocol. The transformants were plated on LB-agar with ampicillin selection and grown overnight at 37°C. Grown colonies were screened with colony PCR using CP-F and UnivR primers and KAPA2G Robust HotStart Kit (Agilent) according to the following protocol:

Component	Volume [µl]	Final concentration/amount
5X Buffer B	2	1x
10 mM dNTP	0.2	200 µM
10 µM CP-F	0.3	300 nM
10 µM UnivR	0.3	500 nM
5 U/µl KAPA2G	0.06	0.3 U
resuspended colony	1	/
H2O	6.14	/

Temperature	Time	Step
95 °C	5 min	hold
95 °C	30 s	
55 °C	15 s	30 cycles
72 °C	1.5 min	
72 °C	5 min	hold

Sanger sequencing of the selected clone confirmed correct sequence of the PVY-coding part and correct in-frame insertion of GFP coding sequence.

Constructed PVY-N605(123)-GFP was coated onto gold microcarriers and used for *Nicotiana clevelandii* bombardment with gene gun (BioRad) according to (Stare *et al.*, 2020). Briefly, 50 µg of constructed PVY-N605(123)-GFP plasmid was isolated from overnight cultures using GenElute Plasmid MiniPrep Kit (Sigma-Aldrich) and used to coat 6.25 mg of gold microcarierrs (0.6 µm) to prepare gene gun bullets according to the manufacturer's protocol. The bullets were used for particle bombardment with Helios® gene gun (BioRad) of 3 weeks old plants at 200 psi.

Primer Name	Sequence (5' → 3')
GFPF40	**GAATTTGAGTGCGATACTTATGAAGTGCACCATCAAGGA**ATGGTGAGCAAGGGCGAGGAGCTGTTC
GFPR40	**CATCCTTTTTAGTGCTTCCTCCTGCATCGATTGTGTCATT***GCCCTGGTGATGAAC*CTTGTACAGCTCGTCCATGCCGAGAGTGATC
CP-F	CTGAAATGATGGTTGCCTTG
UnivR	TGGCGAGGTTCCATTTTCA

Primers used for the construction of PVY-N605(123)-GFP infectious clone. Bolded sequence of GFPF40 primer is complementary to the 3'-end of NIb coding sequence and underlined sequence to the 5'-end of GFP coding sequence. Bolded sequence of GFPR40 primer is complementary to the 5'-end of CP coding sequence and underlined sequence to the 3'-end of GFP coding sequence. Sequence in italic codes for 5 additional aminoacids forming the protease recognition site – VHHQ*G.

References

Bond SR, Naus CC. **2012**. RF-Cloning.org: An online tool for the design of restriction-free cloning projects. *Nucleic Acids Research* **40**: 209–213.

Bukovinszki Á, Götz R, Johansen E, Maiss E, Balázs E. **2007**. The role of the coat protein region in symptom formation on *Physalis floridana* varies between PVY strains. *Virus Research* **127**: 122–125.

Karimi M, Inzé D, Depicker A. **2002**. GATEWAY™vectors for *Agrobacterium*-mediated plant transformation. *Trends in plant science* **7**: 193–195.

Rupar M, Faurez F, Tribodet M, Gutierrez-Aguirre I, Delaunay A, Glais L, Kriznik M, Dobnik D, Gruden K, Jacquot E, *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.

Stare K, Coll A, Gutiérrez-Aguirre I, Tušek Žnidarič M, Ravnikar M, Kežar A, Kavčič L, Podobnik M, Gruden K. **2020**. Generation and in Planta Functional Analysis of Potato Virus Y mutants. *Bio-Protocol* **10**: 1–19.

Video S1: Epidermal chloroplasts cluster around the nucleus in potato plants in response to PVY infection. We observed clustering around the nucleus (asterisks) in the cell adjacent to the cells in which we observed PVY-GFP multiplication (arrows). See separate file.