

Prosystemin coordinates multiple defense responses in tomato

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In *Solanaceae*, a family of related peptide hormones called systemins are involved in the activation of defense genes in response to wounding and herbivory¹. A primary wound signal is an 18-aa oligopeptide, systemin (Sys), which is located at the C-terminus of a cytosolic precursor protein of 200-aa, prosystemin (ProSys), and released from its precursor following wounding with an unknown mechanism. Recent studies indicated that Sys up-regulates locally the biosynthesis of Jasmonic Acid (JA)², the molecule responsible of the systemic wound signaling in tomato. ProSys involvement in tomato response to chewing insects has long been described, but little is known about its role in modulating defence responses to phloem-feeding insects. The feeding habits of these herbivores is very different from chewing larvae, and cause little mechanical damage to plant tissues. Furthermore, as plant response to stress is regulated by a number of coordinated and interconnected pathways, it is interesting to investigate the role of systemin in the activation of plant response to other biotic stresses such as phytopathogenic fungi. The involvement of systemin in the systemic activation of coordinated defence responses has been recently proposed showing that *ProSys* overexpression in tomato upregulates genes involved in the production of volatile compounds that attracts herbivore natural enemies³.

Our aim was to investigate if systemin-mediated response in tomato, influences the performance of the phytopathogenic fungus *Botrytis cinerea* and of the aphid *Macrosiphon euphorbiae*. We showed that the constitutive overexpression of *ProSys* and of a mutated precursor protein lacking the Sys cod-

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ing region, is involved in tomato resistance against fungi and aphids.

Materials and methods

Transgenic tomato (*Solanum lycopersicum* cv. 'Red Setter') plants were obtained by *Agrobacterium tumefaciens* mediated transformation using the binary vector pRSYS, carrying the entire *ProSys* cDNA, or the binary vector pRSYSdel, with a truncated *ProSys* cDNA that lacks the Sys coding region. The coding sequences of the transgenes are under the control of the 35S RNA CaMV promoter. Transgenic and untransformed plants were grown in sterilized soil and maintained in controlled environmental chambers for 3 weeks from sowing at 25°C with a photoperiod of 16:8hr light/dark. Transgene presence was verified by PCR (Table I) targeting *ProSys* and *RbcS* terminator sequences.

Total RNA was prepared from leaves by a phenol/chloroform extraction and a lithium chloride precipitation and treated with RNase-free DNaseI (Invitrogen) to remove residual genomic DNA. First-strand cDNA was synthesized using Revertaid first strand-cDNA synthesis kit (Fermentas) and controlled by

Table I.—Primer sequences and amplification conditions.

	Sequence (5'→3')	PL (bp)	AL (bp)	Gene	Accession Number	T _m ¹	Cycle	NR
PC-BBSBB Fw	GGGAGGGTGCCTAGAAATA	20	110 ²	Proslys	M84801.1	60	30 sec a 95°C	30
PC-BBSBB Rv	TTGCATTTTGGGAGGATCAC	20	717 ³		M84800.1 (McGurl <i>et al.</i> 1992)	58	30 sec a 51°C 2 min a 72°C	
Pc-LeEf Fw	AAGCTGCTGAGATGAACAAG	20	687 ⁴	<i>EF1-α</i>	X14449.1	58	45 sec a 94 °C	25
Pc-LeEf Rv	GTCAAACCACTAGGGCCAAA	20	767 ⁵		X53043.1 (Shewmaker <i>et al.</i> 1990)	54	45 sec a 53 °C 45 sec a 72 °C	
MCOP RbcS Rv	TTGTCGAAACCGATGATACG	20	1086 ⁶ 1607	RbcS	M21375 (Hunt <i>et al.</i> , 1988)	58	BBSBB condition	

amplifying the cDNA of the *Elongation Factor1-α*, a constitutively expressed gene. Primer were designed to be able to detect contaminant genomic DNA as they anneal on adjacent exons (Table I). To quantify prosystemin expression, real time RT-PCR was performed using a Rotor Gene 6000 machine (Corbett Research) using primers annealing on exons 10 and 11 for RSYS plants, and primers annealing on exons 9 and 10 for RSYSdel plants (Table I). Transgene expression was quantified with $\Delta\Delta C_T$ method using the Sybr Green chemistry. Analysis was carried out on three biological replicates of each selected transgenic genotype in comparison to control untransformed plants.

Conidia from the fungus *Botrytis cinerea* were produced by growing the fungus at 20°C on Potato Dextrose Agar. Fungal cultures were suspended in water, filtered to remove hyphal fragments and diluted to a 10⁶ propagules/ml concentration. Ten μ l of this suspension were spotted three times on each tomato leaves and each spot was marked by a black pen; the major and minor axes of necrotic areas

were measured using a digital caliber every 3 days on four plants per genotype.

The aphid *Macrosiphon euphorbiae* was continuously reared on *Solanum lycopersicum* cv. 'San Marzano' in a growth chamber at 20±1°C, 65±5% RH, 18:6hr light/dark. For the bioassay, forty RSYS and 'Red Setter' plants were infested with a newly born first instar nymph. The presence of aphid and of exuviae were monitored daily.

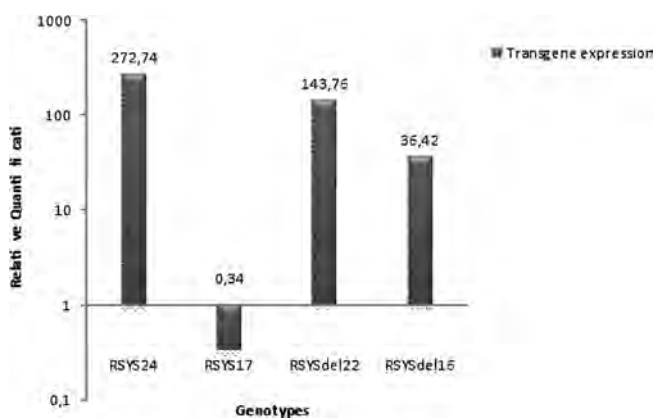


Figure 1.—Relative quantification of prosystemin expression by Real Time RT-PCR. The graph displays the prosystemin relative quantity (RQ; $RQ=2^{-\Delta\Delta C_T}$), shown on a linear scale relative to the calibrator genotype (Red Setter) in RSYS and RSYSdel transgenic lines. Three plants were used for each genotype and each PCR reaction was done in duplicate.

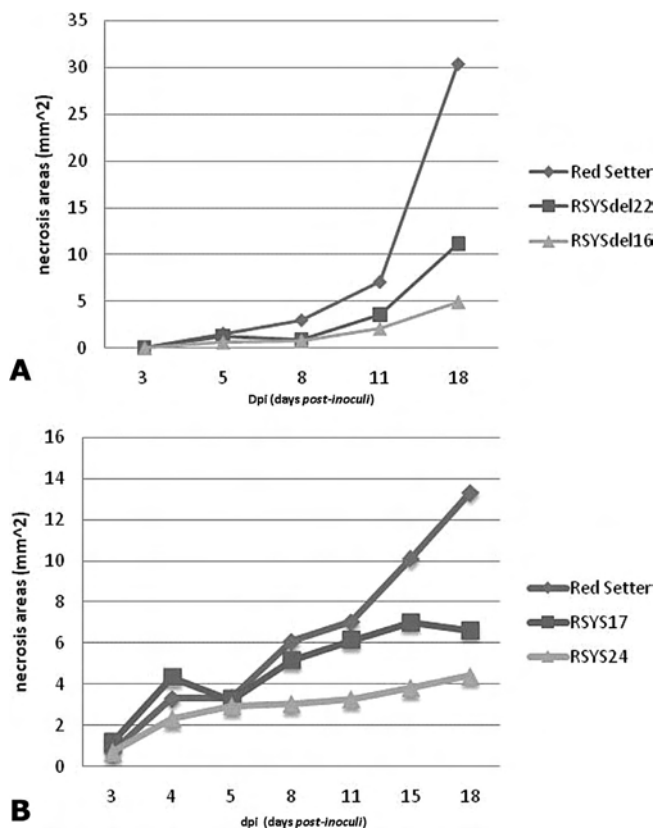


Figure 2.—Bioassay against *Botrytis cinerea*. A: necrosis areas calculated on RSYSdel plants (lines 22 and 16) in comparison to 'Red Setter' at 3,5,8,11 and 18 days *post-inoculi*. B: necrosis areas calculated on RSYS plants (lines 17 and 24) in comparison to 'Red Setter' at 3,4,5,8,11,15,18 days *post-inoculi*.

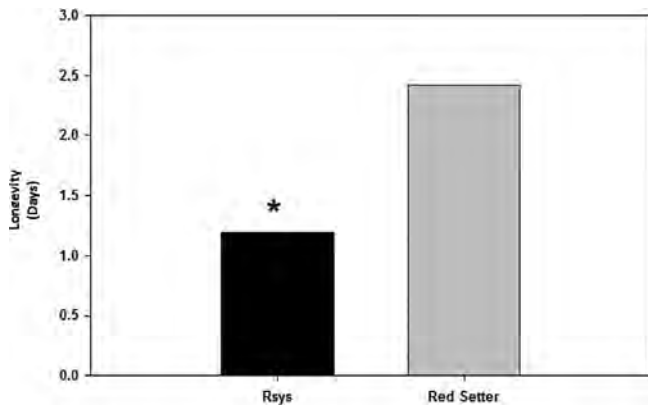


Figure 3.—Aphid bioassay. Aphid longevity measured on RSYS plants (line 24) in comparison to 'Red Setter' plants. *:p<0,05 (t-Student's test).

Results and discussion

Plant genetic transformation was carried out with the pRSYS or pRSYSdel plasmids and allowed the regeneration of 65 and 88 putative transformed lines, respectively. PCR analysis identified 17 and 54 transgenic lines, respectively. The level of expression of *ProSys* gene was quantified by real time PCR. RSYS and RSYSdel plant populations show different expression levels, as expected, which ranged from 0,2 to 90. According to *ProSys* expression level (Figure 1), two RSYS (17, 24) and two RSYSdel (16, 22) transgenic lines were selected for bioassays to evaluate resistance against the phytopathogenic fungus *B. cinerea* and the aphid *M. euphorbiae*.

Figure 2 shows the results of the bioassay with *B. cinerea*. Both RSYS and RSYSdel plants showed smaller necrosis than 'Red Setter' plants. RSYS17 plants show an intermediate behavior between RSYS24 and control plants.

Transgenic plants were also assayed with the aphid *M. euphorbiae*. The maximum intrinsic rate of population increase (*rm*) was not calculated because of the inability of the aphid strain to reproduce on tomato cv. 'Red Setter'. Aphids on RSYS plants had a significantly reduced longevity in comparison to that showed on the control plants (Figure 3), suggesting that transgenic lines are more resistant to this aphid.

In conclusion, our study suggests that *Sys* is involved in the protection of tomato plants against phytopathogenic fungi and floem feeding pests. In addition, we showed that the N-terminal region of the *ProSys* precursor is involved in the activation of defense against fungi, contrasting previous hypothesis about the role of the precursor protein⁴. It is possible that the activation of JA due to the constitutive expression of *ProSys* in transgenic plants allows plants to be pre-adapted to different type of stresses⁵. Future transcriptomic work will verify this hypothesis.

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