# Systemin-inducible defence against pests is costly in tomato

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## Abstract

The possible costs of inducible defences against pests were evaluated in tomato. To activate inducible resistance traits, we used transgenic plants that over-expressed the systemin precursor (prosystemin). The constitutive expression of the prosystemin, which is normally induced by herbivores in tomato, allowed the measurement of the impact of induced defences in a pest-free environment. The results showed that the continuous activation of traits that are normally induced by pests should be costly, affecting the growth, physiology and reproductive success of tomato plants.

*Additional key words*: insect resistance, jasmonic acid, *Lycopersicon esculentum,* prosystemin.

## Introduction

Plants use a wide range of physical and chemical defences to protect themselves from herbivores and pathogens. The majority of chemical compounds involved in resistance against biotic stress is inducible (Walling 2000). It is intriguing that plants have evolved inducible resistance mechanisms, although inducible defences are intrinsically inferior to constitutive defences. With the former, plants display a much lower level of protection during the time-gap between the first attack and the onset of the response. Such apparent inconsistency has been explained by proposing that the production of defensive compounds is costly and hence, in the absence of recurring and frequent attacks, represents an unnecessary use of resources. However, alternative theories, focusing on the co-evolution between plants and their enemies, have also been discussed (Heil *et al.* 2002, Brown 2003).

In tomato, it is long known that insect herbivory induces the accumulation of defence proteins in both damaged and undamaged distal leaves (Chen *et al.* 2005). Considerable progress for the study of the cost of inducible defences against pests has been made using chemical elicitors. Specifically, jasmonic acid (JA) has been successfully used to stimulate plant defense against herbivorous insects (Baldwin 1998, Thaler 1999, Redman *et al.* 2001, Heil *et al.* 2002, Kessler *et al.* 2002). This approach offers the advantage of uncoupling the cost of induced responses from the less controllable pest damage. On the other hand, chemicals are frequently used at concentrations well over the physiological conditions, which may produce secondary (*i.e.* pleiotropic, toxic, *etc.*) effects (Baldwin 2001, Heil *et al.* 2002, Hui *et al.* 2003). For instance, phenotypic effects in plants after JA treatment are evident only at high doses (Redman *et al.* 2001). Furthermore, it is difficult to ensure a uniform and constant distribution of the elicitors throughout the plants with chemicals that are exogenously applied or are volatile (Heil *et al.* 2002).

Alternatively, the cost of defence can be determined between plants that differ only in the genes that control the expression of resistance traits. Gain-of-function mutants, including transgenic lines that constitutively express inducible resistance genes, allow a determination of the cost of inducible defence genes (Cipollini 2006, Heil *et al.* 2002). However, to circumvent possible pleiotropic or non-physiological effects from strong transgenic expression, transgenic lines need to be appropriately selected. While the study of the cost of a

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*Abbreviations*: ABA - abscisic acid; CaMV - cauliflower mosaic virus; JA - jasmonic acid; PCR - polymerase chain reaction.
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single resistance gene can be performed exploiting its over-expression (Brown 2003, Heidel *et al.* 2004), to evaluate the consequences of inducible defences, it is necessary to genetically manipulate the plant endogenous signals that activate inducible defence pathways (Cipollini 2006).

The present study aimed to investigate the cost of inducible defences against pests in tomato as it is not only an important crop, but its responses to herbivores (and the signalling pathways that generate these responses) have been intensively studied. We employed plants that were transformed to constitutively express the prosystemin polypeptide (McGurl *et al.* 1994). This protein is proteolytically cleaved to release the 18-amino acid systemin peptide, which activates the transcription of several defence genes. Although the role of systemin as a long-distance signal is probably limited (Schilmiller *et al.* 2005), a number of studies have indicated that systemin is a primary molecule to activate inducible responses against wounding (McGurl *et al.* 1992, 1994, Ryan *et al.* 2003). Consequently, we had the opportunity to investigate the costs of the inducible defences against herbivores in tomato for the first time in the absence of pests, leaf area-removal or exogenous application of a chemical elicitor.

## Materials and methods

Transgenic tomato (*Lycopersicon esculentum* L. syn. *Solanum lycopersicum* L.) overexpressing the *prosystemin* cDNA under the control of the constitutive 35SRNA CaMV promoter (35S::prosys) and their wild-type were described in detail by McGurl *et al.* (1994). Plants were grown from seed in plastic pots in a pest-free glasshouse without supplemental lighting. Plants were watered as needed, treated with the *Miracle-Gro Pro* fertilizer every two weeks and support was provided with stakes and ties as plants grew. Plants were relocated every two weeks in random fashion to minimize micro-environmental effects. Data were collected from 35 plants per treatment and the genetic identity of the plants was confirmed by PCR at the end of the growing cycle (data not shown).

Wounding experiments were performed using 21-d-old plants. Lower leaves of wild-type plants were wounded with a haemostat perpendicularly to the main vain. After 12 h, wounded leaves were harvested to monitor the local response, while unwounded leaved were collected to assess the systemic induction of the *prosystemin* gene. Leaves of unwounded wild-type and 35S::prosys plants served as reference. The isolation of total RNA and the Northern blot were carried out using already described procedures (Van Blokland *et al.* 1998, Corrado *et al.* 2008). Filters were hybridized with the $^{32}$P-labelled *Hin*d III fragment of the pPS plasmid (McGurl *et al.* 1992), corresponding to the *prosystemin* cDNA. After washing, membranes were exposed to a phosphorimager plate and data were read out with the *Typhoon 9400* imager (*GE Healthcare*, Milano, Italy) using the *ImageQuant v 5.2* software (*Molecular Dynamics,* Milano, Italy).

For each plant, stalk length was measured from the soil level and the internode length every two weeks from 51 d after sowing (when stalk length started being measurable) to 177 d after sowing (plant senescence). On the same dates, the number of nodes and the floral buds produced were also counted. Furthermore, the days of appearance of completely open flowers and of fruit skin veraison were registered. Fruits were harvested when they appeared fully red. All seeds were then removed from each fruit, rinsed, counted, and dried on paper towels for subsequent germination experiments. Seeds were sterilized in a 3 % sodium hypochloride solution for 20 min, washed three times with sterile distilled water and left in the dark at 24 °C for one week on Murashige and Skoog medium supplemented with 20 g dm$^{-3}$ of sucrose and solidified with 8 g dm$^{-3}$ plant agar (*Duchefa*, Milano, Italy).

$CO_2$ assimilation rate, stomatal conductance, and intercellular $CO_2$ concentration were measured on two mature leaves of 8 plants of wild-type and 35S::prosys genotypes. Measurements were taken 60 d after sowing at midday under light saturating conditions (irradiance > 1500 μmol m$^{-2}$ s$^{-1}$). All measurements were performed with a portable gas exchange analyzer (*LCA4*, *ADC*, Hoddesdon, UK).

Fully red berries from control and transformed plants were cut longitudinally in four parts, frozen in liquid nitrogen following seeds removal and stored at -80 °C. Protein extraction, using a phenol-based method, and 2-D electrophoresis were performed as previously described (Rocco *et al.* 2006). Briefly, IPG strips (17 cm, pH 3 - 10, *ReadyStrip*, *Bio-Rad*, Hercules, CA, USA) were rehydrated overnight with 0.3 cm$^{3}$ of IEF buffer containing 9 M urea, 4 % (m/v) *CHAPS*, 0.5 % (v/v) *Triton X-100*, 20 mM dithiotreitol (DTT), 1 % (m/v) carrier ampholytes pH 3 - 10 (*Bio-Rad*), and 300 μg of total proteins. Proteins were focused using a *Protean* IEF cell (*Bio-Rad*) at 12 °C, applying a total of 54 kVh. After focusing, proteins were first reduced by incubating the IPG strips in 1 % (m/v) DTT and then alkylated with 2.5 % (m/v) iodoacetamide in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30 % glycerol, 2 % sodiumdodecyl sulphate). Electrophoresis in the second dimension was carried out on 12 % polyacrylammide gels (18 × 17 cm × 1 mm) with the *Protean* apparatus (*Bio-Rad*), with 120 V applied for 12 h. Samples were run in triplicate. 2-DE gels were stained with colloidal Coomassie G-250 and scanned using a *GS-800* calibrated densitometer (*Bio-Rad*). Image analysis was performed using the *PDQuest* software (*Bio-Rad*). Spot detection and matching between gels were performed automatically, followed by manual matching. A two-fold change in normalized spot densities was considered indicative of a differentially expressed polypeptide ($P < 0.05$)

One-way *ANOVA* was used to study the significance of the effect of genotype on the number of flowers per plant, time of appearance of first flower and first five flowers, time of fruit skin veraison, number of fruit per plant, fruit mass, number of seeds per fruit, seed germination, $CO_2$ assimilation rate, stomatal conductance, intercellular $CO_2$ concentration. Genotype, time, and genotype × genotype effects on stalk length, number of nodes, and internode length were evaluated with repeated measures analysis procedures. All statistical analyses were performed with *SPSS* software (*SPSS Inc*., Chicago, Illinois, USA).

## Results

To assay the implication of the constitutive prosystemin over-expression in the transgenic plants, leaves of the wild-type plants were wounded, and the expression of the *prosystemin* gene analysed by Northern blot. As expected, an easily detectable amount of the *prosystemin* transcript is present in both local and distal unwounded leaves only after wounding (Fig. 1), confirming that the *prosystemin* gene is systemically activated after damage (McGurl *et al.* 1992). Furthermore, the data suggest that the constitutive expression of the *prosystemin* cDNA occurs at a physiologically relevant level in the transgenic lines. The result is consistent with the evidence that transgenic plants, in the absence of damage caused by larvae, accumulate defence proteins (*i.e.* proteinase inhibitors I and II) in amounts similar to those of damaged control plants (McGurl *et al.* 1994).

One advantage of using transgenic technology to assess the cost of inducible defences is to have a continuous activation of responses throughout the whole life of the plant, thus allowing repeated measurements without the need for recurrent treatment with chemical elicitors. The repeated measures analysis detected significant effects of genotype, time and genotype × time interaction on stalk length. 35S::prosys plants showed a reduced growth during the elongation time, but at the end of the growing period, when plant were pot-bound, stalk lengths were not significantly different (Fig. 2). Genotype, time, and genotype × time interaction significantly also affected internode length. Internode length in the 35S::prosys plants was significantly shorter than controls until differences in stalk length were not significant. The data indicated that, when present, length differences were mainly due to reduced internode elongation. Transformed plants produced a greater number of buds than wild-types from the early stages of growth (Fig. 2). 35S::prosys plants were significantly delayed in the appearance of the first and of the first five flowers by around 10 and 6 d, respectively, compared to controls ($P < 0.01$). Differences between genotypes were not significant in the time of fruit skin veraison.

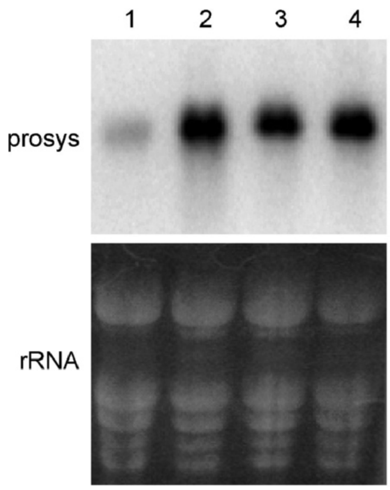


Fig. 1. Northern blot analysis of the induction of the *prosystemin* gene (prosys). 1 - unwounded leaves of wild-type plants, 2 - wounded leaves of wild-type plants (local response), 3 - unwounded distal leaves of wounded wild type plants (systemic response), 4 - unwounded 35S::prosys transgenic plants. Ethidium-bromide stained gel of the ribosomal bands of total RNA (rRNA), shown as loading control.

Overall, the data indicated that the slow-growing plants over-expressing the *prosystemin* gene flower later than control despite producing a higher total number of flower buds. As the breaking of the fruit is not delayed, the data also indicated a reduction of the interval between the flowering time and the maturity of the fruits.

The fitness of a genotype cannot be easily directly quantified. As tomato is a highly selfing species, we considered the number of fruits, their mass and the number of seeds as the main parameters. The plants over-expressing the prosystemin protein produced a lower number of seeds per fruit but differences were not found in the fruit number and mass (Table 1). In addition, the ability of the seeds to germinate was not significantly different (Table 1).

It has been previously shown that prosystemin over-expression alters the 1D electrophoretic profile of soluble proteins from leaves (Bergey *et al*. 1996). Taking into account the reduced number of seeds per fruit in transgenic plants, we wanted to investigate the impact of the prosystemin over-expression in fruits, as it is known that the response to biotic stress can significantly alter their properties (Anand *et al*. 2009).

Proteins from mature tomato berries from control and transformed plants were extracted and subjected to 2-D electrophoresis. Comparison of the protein profiles (Fig. 3), carried out by software-assisted densitometric analysis of resolved gels, indicated the presence of qualitative and quantitative variations. Average proteo-

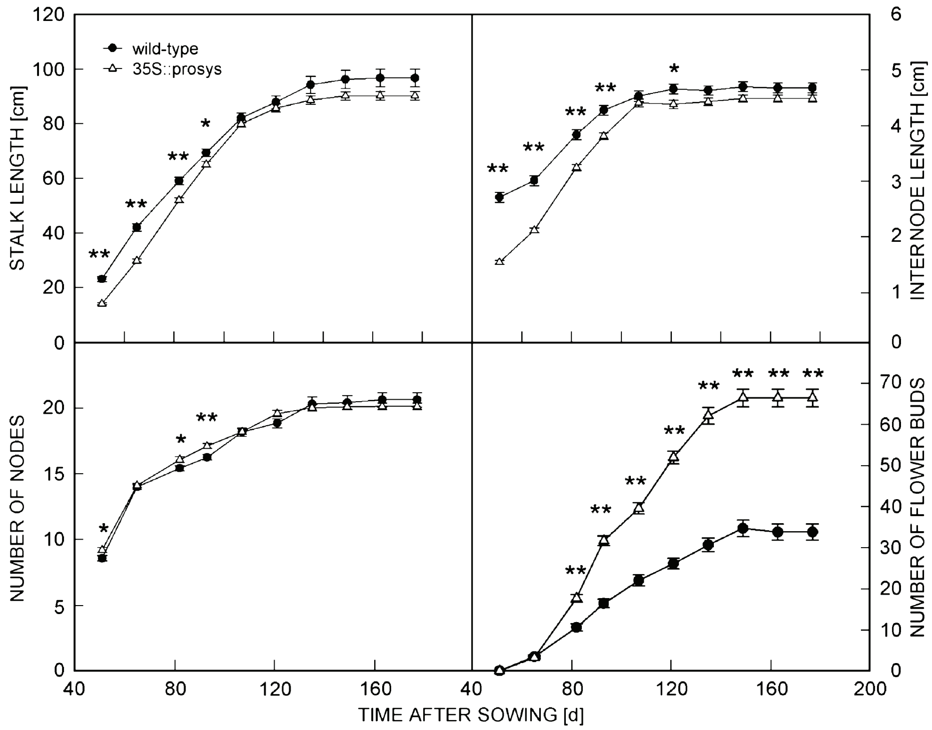


Fig. 2. Stalk length, internode length, number of nodes per plant, and number of flower buds per plant of wild-type and 35S::prosys tomato plants from the day with measurable stalk length (51 d after sowing) until senescence (177 d after sowing). *Asterisks* indicate significant differences between genotypes according to one-way *ANOVA* (* $P < 0.05$, ** $P < 0.01$). *Bars* represent ± SE, $n = 35$.

Table 1. Number of fruits per plant, fruit mass, number of seeds per fruit, percentage of seed germination, $CO_2$ assimilation rate, stomatal conductance, and intercellular $CO_2$ concentration of wild-type and 35S::prosys plants. The significance of the genotype effect on the seven parameters is also reported by *P*-value.

| Genotype | Fruits [plant$^{-1}$] | Fruit mass [g] | Seeds [fruit$^{-1}$] | Germination [%] | $CO_2$ assimilation rate [μmol m$^{-2}$ s$^{-1}$] | Stomatal conductance [mmol m$^{-2}$ s$^{-1}$] | Intercellular $CO_2$ conc. [μmol mol$^{-1}$] |
|---|---|---|---|---|---|---|---|
| Wild-type | 8.33 | 34.8 | 42.9 | 98.2 | 14.4 | 443.3 | 258.3 |
| 35S::prosys | 7.94 | 35.0 | 28.4 | 97.3 | 11.3 | 231.3 | 241.6 |
| *P*-value | 0.77 | 0.96 | 0.041 | 0.537 | 0.016 | 0.013 | 0.131 |

mics maps contained 373 (control) and 347 (35S::*Prosys*) spots respectively, with a 72 % degree of similarity (250 matched spots). Statistical evaluation of relative spot densities highlighted 37 spots whose amounts showed at least a two-fold increase in the transformed berries, and 94 spots showing a corresponding decrease.

Finally, $CO_2$ assimilation rate and stomatal conductance were significantly lower in 35S::prosys than in wild-type plants, whereas genotype did not significantly affect and intercellular $CO_2$ concentration (Table 1).

## Discussion

The evaluation of the cost of inducible defences against pests has given apparently conflicting results. Specifically, the elicitation of defensive proteins in tomato using chemical inducers generated different findings. In a greenhouse study, only high-dose foliar spray of JA caused plants to produce fewer, larger fruits with an overall reduction of the number of seeds per plants, while low-dose treatment resulted in delayed fruit-set and fewer seeds per unit of fruit mass (Redman *et al.* 2001). However, a similarly designed field study found no effect on fruit production (Thaler 1999) and another greenhouse study with tomato using chitosan as elicitor found no fitness effects (Brown 1988). Some authors discussed these discrepancies considering the technical aspect related to the use of chemical elicitors and the possible influence of abiotic factors on the magnitude of

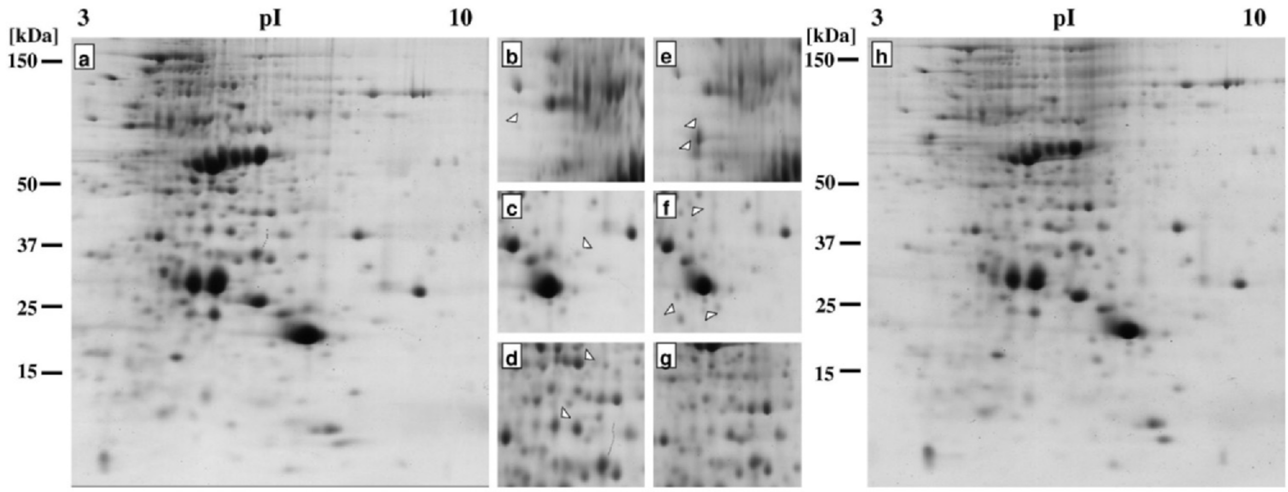


Fig. 3. 2-DE maps of proteins of fruits from the wild-type (*a*) and 35S::prosys plants (*h*). The molecular mass marker *lane* is indicated on the left side of each map and the pH range used for isoelectric focusing is indicated on the top. Densitometric analysis indicated the presence of 101 differentially expressed spots (2-fold, $P < 0.05$). White triangles in the close-up boxes (*b*, *c* and *d*: wild-type, *e*, *f* and *g*: 35S::prosys) point to some over-expressed protein spots.

the effects of the costs (Heil *et al.* 2002, Dietrich *et al.* 2005). Our results support the model that inducible defences against herbivorous pests are costly, affecting the growth, the physiology and the reproductive success of tomato plants.

The simplest explanation is that 35S::prosys plants invest energy and amino acids to continuously produce proteins toxic to larvae and other related defensive compounds (Chen *et al.* 2005) and, if photosynthesis is not up-regulated accordingly, this should limit the amount of resources available to sustain both vegetative and reproductive sink organs. During the interaction with other organisms, plant source-sink relationships can dramatically change because new sinks (*i.e.* pathogen organisms) are established and photosynthesis may be promoted to sustain their growth (Paul *et al.* 2001). Although similar mechanisms should be present for plant-insect interactions, little is known about it (Gassmann *et al.* 2005). The data indicated that the $CO_2$ assimilation rate of 35S::prosys plants was significantly lower than in wild-type plants. This result implies that in the 35S::prosys plants, source limitation to sink growth organs should be larger than the one directly related to constitutively produce defence compounds. The data are consistent with the evidence that exogenous application of JA decreases photosynthetic rate (Lehmann *et al.* 1995, Metodiev *et al.* 1996, Herde *et al.* 1997).

It is also likely that the transgenic plants produce various systemin-regulated metabolites involved in other physiological and developmental processes necessary for plant protection against herbivores (Narvaez-Vasquez *et al.* 2002). For instance, prosystemin overexpression increased salt tolerance in tomato with a mechanism that was not mediated by the ABA signal transduction pathway (Orsini *et al.* 2010). The greater production of flower buds of the transgenic plants support the proposition that endogenous signals correlated to environmental stress may alter developmental processes (Morris *et al.* 2000, Obregon *et al.* 2001). However, the higher number of total buds produced by 35S::prosys plants did not result in an increased number of mature fruits, possibly because the stressed tomato plants have the ability to selectively allocate their resources towards the production (Thaler 1999). Cultivated tomato plants could receive 15 - 30 % mechanical leaf damage without a negative effect on fruit mass (Welter *et al.* 1989).

Similarly, the analysis of the fruit proteome also indicated that the impact of the prosystemin over-expression is complex. The data suggest that the overproduction of defensive proteins in fruits is accompanied by a significant reduction of the amount of several other proteins. We believe that a mass spectro-metry approach for the identification of differentially expressed proteins could be an informative tool for a future molecular dissection of the cost of inducible defence (Mahmood *et al.* 2009).

Our findings are consistent with studies about single resistance genes in wild herbaceous plants (*i.e.* wild tobacco and *Arabidopsis*; Heil *et al.* 2002), but the cost of inducible defences in our work was evaluated over-expressing a plant signal that activates a number of resistance products (Tian *et al.* 2003, Jackson *et al.* 2004, Zavala *et al.* 2004). A limitation of our approach can be that prosystemin mRNA is produced in all plant tissues, including those in which this gene is not necessarily transcribed in a significant amount (McGurl *et al.* 1992). However, the transgenic tomato of the present study has proved to be an excellent experimental tool in plant biology, used in a number of studies for the elucidation of the systemic activation of the pest-inducible defence in plants (McGurl *et al.* 1994, Constabel *et al.* 1995, Bergey *et al.* 1996, Jacinto *et al.* 1997, Dombrowski *et al.* 1999, Li *et al.* 2003). The prosystemin-over-expressing tomato was also employed to show that constitutive activation of the defence pathways against chewer pests resulted into a significant decrease of the larvae damage (Chen *et al.* 2005), which confirms that an high level of constitutive protection is superior to an inducible defence mechanism. Our results add that, in absence of recurrent and frequent pest attacks, a mutation leading to the constitutive activation of defensive traits that are normally inducible should be counter selected.

In conclusion, the morphological, physiological, molecular and fitness data from tomato plants constitutively expressing inducible defences against pests are most consistent with the existence of costs of resistance. Further research will explore the complex interplay between the cost of resistance, the level of protection and the amount of herbivores present, to assess the resulting fitness costs or benefits experienced by plants in fields.

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