TOMATO PROSYSTEMIN GENE IN OTHER SOLANACEAE

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1. SUMMARY

Systemin, an octadeca-peptide isolated from tomato, is a signalling molecule involved in local and systemic wound response. It regulates the activation of more than twenty defensive genes in tomato plants in response to herbivore attacks. Systemin derives from the C-terminal region of a precursor of 200 amino acids, known as prosystemin. Prosystemin homologues have been found in other Solanaceae species such as potato (*Solanum tuberosum*), black nightshade (*Solanum nigrum*) and bell pepper (*Capsicum annum*), all members of the *Solaneae* subtribe, whereas they were not identified in tobacco, which belongs to the *Nicotianae* subtribe. Tobacco possesses two hydroxyproline-rich peptides released from the same precursor (preproTobHypSys) that show systemin-like activity. However, those peptides and their precursors are not similar in sequence to the tomato prosystemin. Even if it was previously showed that tobacco does not respond to tomato systemin when externally applied, it has been recently found that wild type tobacco cells are sensitive to systemin. Furthermore, a recent finding indicate that the constitutive expression of the tomato prosystemin in tobacco plants increases the expression of a number of proteins involved in plant defence against pathogens and oxidative stress. The aim of this project was to increase the understanding of the possible biotechnological role of the tomato prosystemin, with a particular emphasis to the exploitation of this precursor to increase the endogenous resistance against biotic stress. To this goal, the tomato prosystemin cDNA was expressed in tobacco and potato. Moreover, a mutated prosystemin cDNA lacking the 3' terminal systemin encoding exon was also expressed to understand the possible function of the N-terminal region prosystemin precursor in the activation of the defence response. Transgenic tobacco plants expressing either of the prosystemin genes were already available. Transgenic potato plants were obtained after an *Agrobacterium*-mediated plant transformation. Tobacco and potato transformants were characterized and a group of genes involved in plant response against biotic stress was analysed by Real Time PCR. The modification in gene expression registered in MZ transgenic plants (expressing the tomato prosystemin cDNA) and PRO8 plants (expressing the deleted prosystemin) showed that *HSP*, *GST*, *Pin II* and *TobHypSys*, all related to plant response to stress, are over-expressed in tobacco transformants. Among the gene tested, *GST* gene was over-expressed only in MZ transgenic plants. Furthermore, in tobacco, the over-expression level induced by prosystemin of *HSP* and *Pin II* is similar to the effect of wounding. A bioassay with the pathogenic fungus *Botrytis cinerea* showed a moderate increase in resistance in the PRO8 tobacco plants. The expression of prosystemin gene in potato does not affect pathogen-related genes as *GluB2* and *PR1b* nor the defence-related potato endogenous systemins, *PotProsys* and *prePotHypSys* genes. A effect was observed for *Lox3* that was over-expressed in potato in both MZ and PRO8 transgenic plants. These data imply that the modification in gene expression in tobacco and potato is not only due to the *Sys* sequence and that the N-terminus is also involved.

2. RIASSUNTO

Le piante e gli insetti coesistono da milioni di anni generando diverse interazioni. Queste possono essere di mutuo beneficio oppure antagoniste. In particolare, queste ultime implicano la predazione delle piante da parte degli insetti fitofagi e la risposta di difesa della pianta. La difesa della pianta può essere di tipo costitutivo cioè sempre presente nella pianta. Tale difesa comporta un costo metabolico da sostenere continuamente nel tempo indipendentemente dalla presenza di un insetto dannoso. Per tale motivo la pianta ha sviluppato delle risposte di difesa indotte dall'attività alimentare degli insetti (Kessler e Baldwin, 2002).
Oltre ad essere costitutivi o inducibili, i meccanismi di difesa della pianta possono essere diretti ed indiretti.
Con la difesa diretta, la pianta agisce influenzando la fisiologia degli insetti fitofagi e il loro comportamento. Le difese dirette possono essere distinte in meccaniche e biochimiche. Le prime consistono in modificazioni morfologiche come spinosità, la durezza tegumentale, la presenza di peli ghiandolari, la secrezione di lattice, cere, resine etc., che essendo barriere strutturali, impediscono agli insetti erbivori di accedere alle sostanze nutritive (Boege, 2005). Le difese biochimiche prevedono la sintesi di nuovi composti in grado di ostacolare i processi digestivi e di assorbimento dei nutrienti degli insetti fitofagi (Felton e Gatehouse, 1996; Felton, 2005). Queste difese includono: inibitori di proteasi (PI), indotte in seguito a ferita e ad attacco di insetti e in grado di interferire con i loro enzimi digestivi; polifenolo ossidasi (PPO) e lipossigenasi (LOX) che riducono il valore nutritivo del tessuto vegetale provocando l'agglutinazione delle proteine ed attivando l'ossidazione di metaboliti fenolici con produzione di chinoni altamente reattivi; per ultimo, composti tossici tra cui alcaloidi, terpeni, sostanze fenoliche, tannini, saponine, etc (Ryan, 1990; Constabel *et al*., 1995; Ryan, 2000). Tutte queste proteine fanno in modo che l'insetto fitofago assuma meno aminoacidi essenziali, compromettendone la crescita e lo sviluppo (Chen *et al*., 2005).
Le difese indirette consistono nella produzione di sostanze che attraggono predatori e parassitoidi degli insetti erbivori (Howe e Jander, 2007). La pianta stabilisce col nemico naturale del fitofago un rapporto mutualistico sia fornendo nutrimento, ad esempio la produzione di nettare extrafiorale (Pulice and Packer, 2008), sia producendo composti chimici volatili che facilitano l'individuazione della pianta attaccata dall'insetto-preda (Dicke e van Loon, 2000). Oltre alla funzione appena citata i composti organici volatili (VOC) svolgono anche un ruolo difensivo allontanando l'ovideposizione degli insetti dannosi (De Moraes *et al*., 2001) e sono coinvolti anche nella comunicazione pianta-pianta (Farmer, 2001). Le risposte della pianta agli insetti dannosi sono diverse e complesse. Un ruolo centrale però è occupato dalla via metabolica degli octadecanoidi.
La via degli octadecanoidi è indotta da ferita e/o da attacco di insetti fitofagi ed è particolarmente caratterizzata nella famiglia delle Solanaceae. Questa via produce composti volatili C_6 ed acido jasmonico e derivati (jasmonati). I volatili C_6 sono responsabili dell'attivazione di geni di risposta a danno meccanico e risultano avere attività antimicrobica e antifungina ad elevate concentrazioni (Walling, 2000). L'acido jasmonico è una molecola segnale ed è coinvolto nell'attivazione delle difese dirette della pianta; i jasmonati modulano l'espressione di numerosi geni ed influenzano diversi aspetti della vita della pianta, come la crescita, sviluppo e la risposta allo stress biotico ed abiotico (Creelman and Mullet, 1997). La sintesi di componenti della via di trasduzione del segnale rappresenta una strategia di amplificazione del

segnale attraverso la pianta. In pomodoro, uno dei primi eventi nel pathway di trasduzione del segnale che porta alla sintesi di proteine di difesa è il rilascio di sistemina, un ormone vegetale proteico di 18 amminoacidi rilasciato in seguito al taglio di un polipeptide precursore di 200 amminoacidi denominato prosistemina (Pearce *et al*., 1991). La prosistemina in pomodoro è codificata da un gene di 4176 bp che comprende 11 esoni di cui l'ultimo codifica per la sistemina posizionata nella regione C-terminale del precursore (McGurl *et al*., 1992). La sistemina è stato il primo peptide segnale identificato in pianta con funzioni di regolatore genico e di trasduttore del segnale cellulare. Da allora numerosi studi sono stati effettuati per approfondire il ruolo di questo peptide segnale. La sistemina è attiva a livelli straordinariamente bassi, a livelli femtomolari, mentre si accumula a livelli più elevati in risposta a ferita ed a danno meccanico sia localmente che sistematicamente nel resto della pianta (Ryan, 2000).
La sistemina, una volta attivata provoca l'induzione di una cascata di eventi intracellulari coordinati che vanno ad attivare la fosfolipasi A_2 (PLA_2), che a sua volta provoca il rilascio dell'acido linolenico (LA) dai lipidi di membrana, cominciando così la via degli octadecanoidi (Ryan, 2000). Questi eventi prevedono: la depolarizzazione della membrana plasmatica, apertura dei canali ionici, incremento della concentrazione di calcio intracellulare, inattivazione delle ATPase di membrana, attivazione delle MAP chinasi, sintesi di calmodulina e infine attivazione di PLA_2 (Walling, 2000). L'acido jasmonico prodotto si propaga attraverso la pianta ed attiva una serie di geni di difesa, tra cui quelli codificanti per gli enzimi proteolitici coinvolti nell'attivazione della prosistemina con conseguente amplificazione del segnale.
L' importanza della sistemina come molecola segnale nella risposta di difesa è stata studiata in piante transgeniche in cui l'espressione della prosistemina era stata bloccata da una strategia di RNA antisenso (McGurl *et al*., 1992). Le piante transgeniche mostravano un forte indebolimento a livello delle loro risposte sistemiche a ferita ed erano più suscettibili agli attacchi del lepidottero *Manduca sexta*. Dall'altro lato, con la sovraespressione della prosistemina in piante di pomodoro è stata indotta una produzione costitutiva di proteine usualmente sintetizzate dopo uno stress meccanico (McGurl *et al*., 1994). Inoltre, queste piante transgeniche sovraesprimenti il gene della prosistemina mostravano un aumento nella produzione di composti organici volatili (VOC) (Corrado *et al*., 2007). Con questi esperimenti si è dimostrato che la sistemina è coinvolta nella attivazione dei geni di difesa diretta ed indiretta nel pomodoro.
La prosistemina è stata anche espressa in patata (Narváez-Vásquez and Ryan, 2002). L'espressione costitutiva del cDNA della prosistemina di pomodoro, produce alti livelli di inibitori di proteasi nelle foglie come nei tuberi, simile all'effetto della sovraespressione di prosistemina in pomodoro.
Un modello precedente indicava che la sistemina venisse liberata nel floema e trasportata sistematicamente ai tessuti non danneggiati dove fosse in grado di regolare le risposte di difesa indotte da ferita. Da studi condotti sui mutanti di pomodoro emergono nuove evidenze, che suggeriscono che la sistemina agisce a livello locale probabilmente per incrementare la sintesi di acido jasmonico, e che l'acido jasmonico o un altro componente derivato della via degli octadecanoidi, potrebbe servire come molecola segnale.
Ortologhi della prosistemina sono stati trovati in altre piante della famiglia delle *Solanaceae* per esempio patata (*Solanum tuberosum*), peperone (*Capsicum annum*) ed erba morella (*Solanum nigrum*), tutti della *subtribae Solaneae*. Tuttavia la sistemina non è stata individuata nel tabacco, che appartiene alla *subtribae*

Nicotianae. Studi condotti su sospensioni cellulari di tabacco hanno evidenziato la presenza di altri peptidi coinvolti nella risposta di difesa della pianta (Pearce *et al*., 2001). I due polipeptidi del tabacco, peptidi ricchi di idrossiprolina e rilasciati da un unico precursore proteico, sono funzionalmente simili alla sistemina del pomodoro, ma strutturalmente diversi, dato che non presentano similarità di sequenza con la sistemina. Tra l'altro, questi peptidi presentano un'elevata similarità con la sistemina a livello del meccanismo di azione e nel ruolo nella trasduzione del segnale. Per tale motivo sono stati classificati come "sistemine", TobHypSys I e II. Le due sistemine del tabacco derivano da preproTobHypSys-A, un solo precursore di 165 amminoacidi munito di una sequenza segnale. TobHypSys I si trova nella regione N-terminale, mentre TobHypSys II nella regione C-terminale. Il ruolo di questi peptidi nella risposta di difesa della pianta è stato documentato da diversi esperimenti. Rocha-Granados *et al*., (2005) hanno dimostrato che l'espressione di preproTobHypSys-A (ppTobHS-A) è indotta da ferita, attacco di insetti fitofagi ed elicitori implicati con insetti fitogagi. Inoltre, piante di tabacco sovraesprimenti il gene TobpreproHypSys-A hanno una migliore resistenza contro la larva di *Helicoverpa armigera*, forse dovuta alla sovraespressione di inibitori di proteasi (PI) e polifenolo ossidasi (PPO) nelle piante transgeniche (Ren and Lu, 2006). In più, è stato anche indicato che l'espressione della prosistemina e del precursore TomHypSys nella risposta a ferita è importante per generare un forte segnale sistemico di geni di difesa, stimolando la via degli octadecanoidi e la biosintesi dei jasmonati (Narváez-Vásquez *et al*, 2007).
Il presente lavoro di ricerca si è basato sullo studio degli effetti dell'espressione del gene della prosistemina di pomodoro e del medesimo gene deleto della porzione codificante la sistemina in due specie di Solanaceae, tabacco e patata. Lo scopo dell' attività di ricerca è stato l'ampliamento delle attuali conoscenze riguardanti la prosistemina ed il suo possibile ruolo biotecnologico con particolare enfasi sull'utilizzo di questo precursore al fine di aumentare la resistenza endogena della pianta contro lo stress biotico in specie diverse dal pomodoro. A tale scopo sono state studiate piante transgeniche di tabacco e patata, i genotipi transgenici MZ presentano l'inserzione di un costrutto contenente un promotore costitutivo CaMV 35S, il cDNA della prosistemina del pomodoro, la sequenza di terminazione del gene *rbcS*, ed il gene neomicina fosfotransferasi, che conferisce la resistenza all'antibiotico kanamicina. I genotipi transgenici PRO8 contengono il medesimo costrutto però con la prosistemina deleta della sua porzione terminale codificante per sistemina. Piante transgeniche MZ e PRO8 di tabacco erano già presenti nel laboratorio, mentre le piante di patata della cultivar Desiree sono state ottenutte tramite trasformazione genetica con *Agrobacterium tumefaciens*.
In seguito è stato monitorato il profilo di espressione di alcuni geni collocati all'inizio e alla fine della via degli octadecanoidi ed anche geni attivati in condizioni di stress. L'analisi trascrizionale è stata condotta tramite Real Time PCR che ha misurato l'espressione genica relativa usando il metodo comparativo dei Ct. I geni scelti per tabacco sono stati: *RNA-binding protein* (*RBP*), *heat-shock protein* (*HSP*), *stromal ascorbate peroxidase* (*SAP*), *glutathione S-transferase* (*GST*), tutti coinvolti nella risposta della pianta allo stress, selezionati da un precedente studio proteomico (Rocco *et al*., 2008) che ha dimostrato che l'espressione costitutiva del gene della prosistemina di pomodoro in tabacco è associata ad un incremento delle proteine coinvolte nella difesa della pianta contro patogeni ed anche nello stress ossidativo. Sono stati ugualmente studiati i geni che codificano per calmodulina (*CaM*), per lipossigenasi (*Lox1*) e per inibitori di proteasi II (*Pin II*); CaM e Lox1 sono due enzimi collocati all' inizio della via degli octadecanoidi, mentre Pin II è una proteina prodotta

alla fine della via. Per ultimo, i geni *TobHypSys I* e *II* sono stati analizzati per studiare una possibile interazione tra la sistemina del pomodoro e le sistemine del tabacco. In seguito, le piante MZ e PRO8 sono state saggiate contro il fungo fitopatogeno *Botrytis cinerea*, agente della muffa grigia, per valutare se le piante transgeniche erano più resistenti all'attacco.
Nelle piante di patata transgeniche è stata verificata l'espressione, tramite Real Time PCR, di geni coinvolti nella difesa della pianta. I geni analizzati sono stati: *pathogenesis-related protein1b* (*PR1b*); *1,3-beta-D-glucanase* (*GluB2*), tutte e due geni coinvolti nella risposta a patogeni. Geni derivati della via degli octadecaniodi: *calmodulina* (*CaM*), *lipossigenasi 3* (*Lox3*), *idroperossido liasi* (*HPL*), tre enzimi collocati all' inizio della via degli octadecanoidi, CaM and Lox3 che coadiuvano per generare 13-idroperosside che poi genererà 12-OPDA, e HLP responsabile della produzione di composti volatili C_6 a partire di 12-OPDA. In fine, sono stati analizzati i geni della prosistemina endogena della patata (*PotProsys 1* and *2*) e il precursore dei glicopeptidi ricchi di idrossiprolina (*prePotHypSys*), tutte e due coinvolti nella difesa.
L'analisi di espressione delle piante transgeniche di tabacco trasformate con il gene della prosistemina (MZ) e della prosistemina deleta della sequenza codificante per l'ormone sistemina (PRO8) ha mostrato che i geni *RBP* e *SAP* non appaiono influenzati dalla prosistemina, mentre i geni *HSP* e *GST* sono sovra espressi nelle piante transgeniche. L'analisi invece di geni coinvolti nella difesa, mostra che la prosistemina non induce l'espressione dei geni *CaM* e *Lox1*, però influenza la trascrizione del gene *Pin II*. Per quanto riguarda i geni *TobHypSys I* e *II*, il livello d'espressione è più alto nelle piante transgeniche confrontate con le piante non trasformate. L'effetto della prosistemina nativa e deleta è simile per tutti i geni analizzati in tabacco, escluso *GST*, dimostrando che il cambiamento nell'espressione genica non è dovuto unicamente alla sistemina. Per valutare l'importanza biologica dell'effetto del gene della prosistemina, il livello d'espressione dei geni sovra espressi *HSP, GST, Pin II* e *TobHypSys* è stato analizzato in piante non trasformate dopo danno meccanico. Dall'analisi di espressione emerge che la sovra espressione di *GST* and *Pin II* indotta per la prosistemina e simile all'effetto che produce il danno meccanico.
Posto che l'analisi di espressione indicava che l'espressione costitutiva della prosistemina di pomodoro in tabacco è associata con un cambiamento nell'espressione di alcuni geni coinvolti nella difesa della pianta, le piante MZ e PRO8 sono state saggiate contro il fungo fitopatogeno *Botrytis cinerea*. Le piante PRO8 hanno mostrato una maggiore resistenza rispetto alle piante controllo, sia in termini di dimensioni sia in termini di tempi di sviluppo delle necrosi. Per le necrosi provocate da *B. cinerea* sulle piante MZ sono stati osservati rallentamenti nello sviluppo rispetto al controllo, anche se non è stato riscontrato un arresto dello sviluppo della malattia. Tali osservazioni hanno evidenziato che la prosistemina causa delle alterazioni del livello di espressione di geni che incrementano la resistenza contro un fungo fitopatogeno.
L'analisi invece delle piante transgeniche di patata MZ e PRO8 mostra che l'espressione dei geni coinvolti nella risposta a patogeni come *GluB2* e *PR1b* non sono indotti dalla prosistemina. L'analisi dei geni coinvolti nella difesa come *CaM, HPL* e *Lox3*, rivela che soltanto *Lox3* è sovra espresso nelle piante transgeniche. Per ultimo, l'analisi della espressione dei geni delle sistemine endogene della patata *PotProsys* e *proPotHypSys* non sono indotti dalla prosistemina del pomodoro. In patata, l'effetto della prosistemina nativa e deleta è simile per *Lox3*, l'unico gene sovra espresso, rafforzando l'ipotesi che la modificazione in espressione genica non

è dovuta soltanto alla sequenza della sistemina e che la regione N-terminale e anche coinvolta nell'attivazione dei geni di difesa.
In conclusione, i risultati mostrati in questo studio indicano che l'espressione costitutiva della prosistemina di pomodoro in tabacco è associata con un alterazione nella espressione di alcuni geni coinvolti nella risposta della pianta come *HSP, GST*, *Pin II* e *TobHypSys*. Inoltre il biosaggio col fungo *B. cinerea* indica che l'espressione della prosistemina produce alterazioni che aumentano la difesa contro i funghi. In sintesi, l'accumulo costitutivo in tabacco di un precursore come la prosistemina è associato con differenze inattese nell'espressione genica e che alterano un carattere della pianta. Invece, in patata, l'espressione della prosistemina del pomodoro pare abbia un effetto molto specifico dato che soltanto *Lox3* è sovra espresso. Analizzando l'espressione genica di tutti i geni studiati nelle piante transgeniche MZ a PRO8, si evidenzia che l'effetto che provoca la prosistemina e l'allele mutato della prosistemina privo dell'esone codificante per la sistemina è similare, indicando un possibile ruolo della regione N-terminale del precursore nell'attivazione dei geni di difesa.
Il lavoro di ricerca dimostra che l'espressione eterologa di precursori di molecole segnale può evidenziare effetti inattesi e, in questo caso, potenzialmente utili ai scopi biotecnologici.

3. INTRODUCTION

3.1. An overview of the plant defence mechanisms against herbivores

In their natural environment, plants encounter numerous biotic and abiotic challenges at once. Each of these environmental stresses activates various signal transduction pathways, so that an effective plant defence response is guaranteed (Ryan, 2000). The plant-insect interaction has existed for millions of years and plants have developed sophisticated defence systems to fight-back insects. As a result, plants must be capable to recognize and prioritize each signalling pathway to have an efficient defence strategy to diminish insect damage and to maintain plant fitness (Karban and Baldwin 1997).

Generally, plants show two types of functional responses to herbivore attack, resistance and tolerance. Tolerance is to reduce the negative effects of herbivore damage on plant fitness without affecting the herbivore, i.e. it decreases the plant fitness cost of herbivore attack. A plant genotype is named tolerant if it can sustain tissue loss with little decline in fitness (Stowe *et al*., 2000). Resistance is the ability of plants to directly or indirectly reduce the amount of damage a plant receives, either by repelling potential herbivores or by diminishing the amount of tissue removed (Tiffin, 2000).

The mechanisms of plant defence are divided into constitutive and induced, yet the two categories overlap to a large extent. Induced defence usually involves systemic induction. Insect herbivores activated induced defences both locally and systemically by signalling pathways that involve systemin, jasmonates, oligogalacturonic acid and hydrogen peroxide (Gatehouse, 2002). The defence response takes place at the site of damage by the insect pest. Subsequently, the defence response occurs all through the plant thanks to signalling molecules that are able to communicate between different plant tissues (Gatehouse, 2002; Ferry *et al*., 2004). The systemic response may result in the production of the same defensive proteins as the local response. A classic example of plant wounding response is the synthesis of proteinase inhibitors in leaves of tomato in response to feeding by larvae of lepidopteran pest species such as *Manduca sexta* (Orozco *et al*., 1993).

A constitutive defence is often the reason that specific plant are resistant to specific insect pests. The constitutive defence can function as (i) a physical barrier such as lignification or resin production; (ii) a biochemical signal recognized by the herbivore such as deterrents of feeding or oviposition; (iii) a toxin, causing membrane disruption, inhibition of transport or signal transduction, inhibition of metabolism, and even disruption of hormonal control of developmental processes in the insect metabolism (Cortesero *et al*., 1999; Gatehouse, 2002; Felton, 2005).

The classic distinction between constitutive and induced defence is often difficult to apply to chemical defences at the molecular level. The end-products or defensive reactions are often the same in constitutive and induced defences in a given plant, as example, the toxin peptides generated in induced defence responses are also accumulated as constitutive defences in some tissues or organs.

Plants use constitutive and induced defences in order to protect themselves from pests, provoking changes in egg deposition, feeding, growth, fecundity, fertility and herbivore development. Every defence mechanism is costly, and the plant must achieve a balance to ensure survival without sacrificing vitality, longevity or reproduction (Walling, 2000). These defences are a set of compounds that function as direct or indirect defences.

3.1.1. Direct defences

Physical barriers or other plant defensive traits that deter arthropods such as a variety of spines, hardened or reinforced leaves, trichomes and the inclusion of minerals into plant tissues are classic direct defences (Hanley *et al*., 2007). However, any morphological or anatomical characteristic that confers a fitness advantage to the plant in the presence of herbivores can be considered a structural defence (Boege, 2005).

Similarly, any compound that provoke repellent, antinutritive, or toxic effects on herbivores are also referred to as direct defences. Defensive metabolites are classified by their mode of action. These are: (i) antidigestive proteins, such as proteinase inhibitors that are inducible after wounding and herbivory. These proteins influence herbivore performance by inhibiting digestive enzymes of attacking pests (Ryan, 1990); (ii) antinutritive enzymes such as polyphenol oxidases that reduce the nutritive value of the wounded plant by cross-linking proteins (Constabel *et al*. 1995); (iii) toxic compounds (e.g., phenolics, alkaloids, terpenoids) which poison generalist herbivores, forcing insects to invest resources in detoxification mechanisms. All these proteins reduce uptake of indispensable amino acid by insects which compromises insect growth and development (Chen *et al*., 2005).

The most studied proteins involved in direct defences in tomato and other Solanaceous plants are proteinase inhibitors (PIs), these proteins are expressed rapidly and systemically in response to wounding. PIs that impair the activity of digestive proteases are perhaps the best example of this type of post-ingestive defence (Ryan, 1990). The PIs capacity to slow herbivore growth depends on accumulation to relatively high concentrations inside the gut lumen of the insect. The negative effect of PIs on herbivore performance is thought to result from a compensatory response by the insect to hyper-produce digestive proteinases which causes a depletion of essential amino acids and has a negative effect on the insect growth and development (Duffey and Stout, 1996).

Decreased growth of herbivores is obviously not fully explained by the action of PIs but involves multiple plant compounds. It is likely that an important factor is the combination of toxic, antinutritive, and antifeedant effects that plants produce (Duffey and Stout, 1996). For example, wound-inducible polyphenol oxidases (PPO) that covalently modify dietary proteins act together with PIs to reduce the nutritional quality and the digestibility of plant food (Constabel *et al*., 1995).

3.1.2. Indirect defences

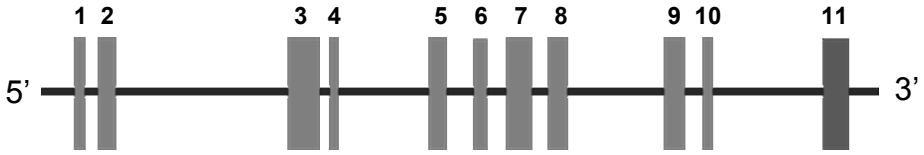
Indirect defence is a plant defensive attribute that protects against herbivory by increasing plant attraction of predators and parasitoids of herbivores (Howe and Jander, 2007). Indirect defences increase the carnivore's hunting success and facilitate the biological control of herbivore populations (Karban and Baldwin, 1997). Indirect protection is mediated by a group of chemical diverse compounds known as volatile organic compounds (VOCs). VOCs are emitted in higher amounts by attacked plants (Dicke and van Loon, 2000).

Plants normally emit volatiles into the atmosphere but the volatile blend changes after wounding and herbivore attack. Volatiles can be influenced by herbivore species, developmental phase, plant age, and type of environmental stress (Parè and Tumlinson 1999).

The VOC response can be very specific, for instance parasitic wasps often use this specificity to locate particular hosts (Turlings and Benrey, 1998). Generalist predators are also attracted by single components of the VOC bouquet, that are usually emitted after the attack of different species of herbivores. Moreover, the VOC emission can function as direct defence by deterring ovipositing herbivores.
Also, extra floral nectaries augment their rate of nectar secretion after herbivore attack, and these carbohydrates and proteins provide nutritional support for predators to increase their foraging rate (Kessler and Baldwin, 2002).
Some volatiles that are commonly emitted by many different plant species include C_6 aldehydes, alcohols and esters (green leaf volatile); indole and methyl salcilic acid; cyclic and acyclic terpenoids (C_{10} and C_{15}), and oximes and nitriles while others are specific to a particular species (Parè and Tumlinson, 1999; Ferry *et al*., 2004). The manipulation of VOC can affect insect resistance as it has been shown that transgenic potato plants that produced low levels of hydroperoxide lyase (HPL), the enzyme involved in green leaf volatile synthesis, were found to support an improved aphid performance and fecundity (Vancanneyt *et al*., 2001).
The production and release of VOCs involves both locally damaged leaves and undamaged distal leaves. However, a VOC specific transmittable signal has not been found in plant yet. Moreover, it has been shown in tomato, that the same signal (the systemin peptide) modulates either direct and indirect response (Corrado *et al*, 2007).

3.2. Systemin peptides in Solanaceae

Systemin, an octadeca-peptide isolated from tomato is the signalling molecule involved in the local and systemic wound response. It derives from the C-terminal region of prosystemin, a prohormone of 200 amino acids (McGurl and Ryan, 1992). The systemin was identified as the primary signal for systemic defence and it is a potent activator of the octadecanoid pathway.
The tomato prosystemin gene is composed of 4526 bp, which includes a 104 bp 5'-untranslated region, a 4176 bp coding region and a 246 bp 3'-untranslated region. The coding region has ten introns and eleven small exons with a dimension between 34 bp and 90 bp. The first ten exons are organised as five pairs and the unpaired eleventh exon encoding systemin is situated at the 3'end (Fig. 1).



McGurl and Ryan, 1992

Fig. 1. The organization of the prosystemin gene. Exons are represented by vertical gray bars numbered 1 to 11. The five exon pairs are 1 plus 2; 3 plus 4; 5 plus 6; 7 plus 8 and 9 plus 10. Exon number 11, in dark gray, represented the sequence encoding systemin, it is located at the 3'end and it is unique when compared to the rest of the sequence of the larger precursor.

The important role of systemin in signalling defence genes has been elucidated by transforming tomato plants with an antisense prosystemin cDNA (McGurl *et al*., 1992). Transformed antisense plants exhibited a severely reduced systemic induction

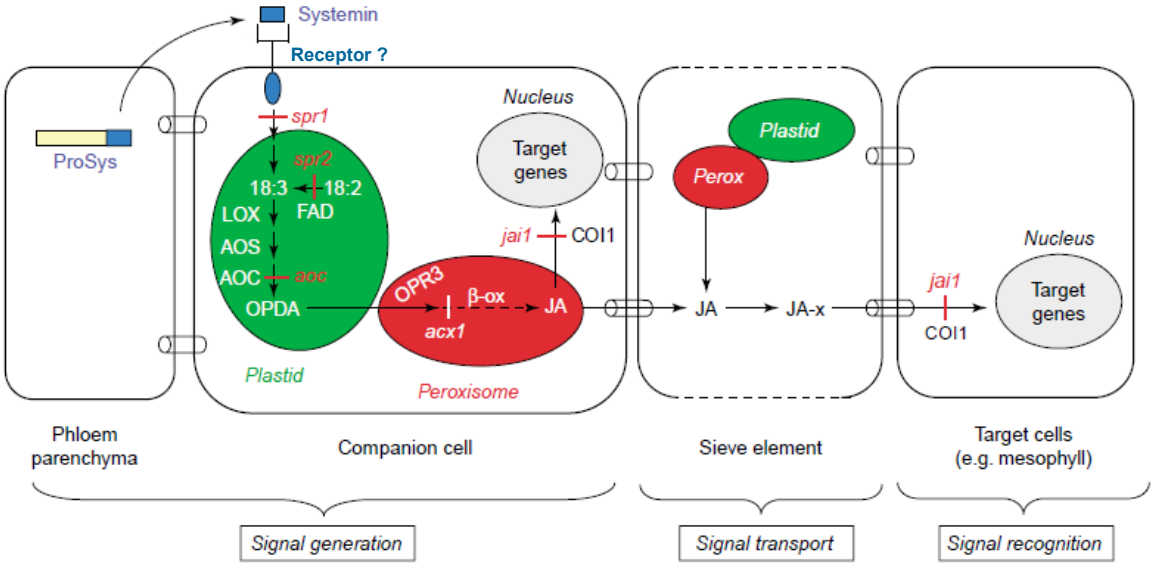
of proteinase inhibitors in response to wounding. Transgenic plants also showed a declined resistance against *Manduca sexta* larvae compared to wild type plants. Moreover, these plants when supplied with external systemin, were able to induce defensive proteins, indicating that they could generate the wound signal mechanism but the signal peptide.

Later on, tomato plants were also transformed with the prosystemin cDNA in its sense orientation (McGurl *et al*., 1994). Surprisingly, transformed plants synthesized wound-inducible defensive proteins in absence of wounding, maybe due to the atypical release of systemin from the precursor. The analysis of proteins accumulated in transgenic plants over-expressing prosystemin resulted in the identification of various systemin-inducible genes, such as (i) defence genes such as serine proteinase inhibitor I and II, cysteine proteinase inhibitor, aspartic proteinase inhibitor, metallo-carboxypeptidase inhibitor and polyphenol oxidase, (ii) signal pathway genes such as lipoxygenase, allene oxidase synthase, calmodulin, polygalacturonase and others; (iii) proteinase genes such as leucine aminopeptidase, aspartic proteinase, cysteine proteinase and carboxypeptidase (Ryan, 2000). The over-expression of prosystemin in tomato also increased the production of volatile organic compounds, implying that the systemin peptide is involved in the activation of both direct and indirect defence genes (Corrado *et al*., 2007).

To verify that systemin is a key signal for the systemic wound response in tomato, grafting experiments were performed with transgenic plants over-expressing prosystemin and wild type plants (McGurl *et al*., 1994). Wild type tomato plants grafted onto the transgenic root stocks accumulated proteinase inhibitors in wild type scions in absence of wounding, meaning that the systemin or a signal generated by systemin was transported from the transgenic root stock to the untransformed scion.

An experiment to determine the systemin transport through the plant was performed by Narváez-Vásquez and others (1995). A synthetic ^{14}C-labeled systemin was applied on fresh wounds, it was distributed in the whole leaf and then transported to the petiole, until reaching the upper leaves. These experiments established that the systemin moves from the wound place to the vascular system via the xylem and then the phloem to be transported to target cells through the plant. However, more recently Li and others (2002) demonstrated that systemin was only involved in the local response. Grafting experiments of non-transformed plants with *spr2* mutants (they do not produce jasmonic acid) and *jai-1* mutants (they produce JA but are insensitive to JA) showed that the jasmonic acid, or a related compound derived from the octadecanoid pathway, might act as a transmissible wound signal. Lee and Howe (2003) confirmed this hypothesis after analysing *spr1* mutants (they are insensitive to systemin) that were unable to accumulate jasmonic acid. These experiments showed that the activation of the jasmonate pathway in response to wounding or prosystemin/systemin is required to generate a long-distance signal whose detection in distal leaves depends on jasmonate signalling (fig. 2).

It is believe that once systemin is released by proteolytic cleavage of the prosystemin and transferred to the phloem by an unknown mechanism, the systemin binds to a receptor on the surface of companion cells and initiate a complex intracellular signaling pathway. This pathway involves the activation of a mitogen-activated protein kinase (MAPK), the rapid alkalinisation of the extracellular medium, the activation of a phospholipase and the release of linolenic acid that is converted into oxylipins such as phytodienoic acid and jasmonic acid that are powerful signals for defence genes (Narváez-Vásquez *et al.*, 1999; Pearce *et al*, 2001; Ryan 2000; Schilmiller and Howe, 2005).



Modified from Schilmiller and Howe, 2005

Fig. 2. Schematic representation of the systemin signalling pathway that activates the octadecanoid pathway in tomato, which generates jasmonic acid (**JA**) by a serial steps of β-oxidation. The systemin peptide derives from the C-terminal end of the prosystemin precursor (ProSys). Modified forms of jasmonic acid (**JA-x**) may possibly play a role in systemic signalling. **spr1 and spr2**, suppressor of prosystemin-mediated responses 1 and 2. **18:3**, linolenic acid. **18:2**, linoleic acid. **FAD**, fatty acid desaturase. **LOX**, 13-lipoxygenase. **AOS**, allene oxide synthase. **AOC**, allene oxide cyclase. **OPDA**, 12-oxo-phytodienoic acid. **OPR3**, 12-OPDA reductase3. **Acx1**, Acyl-CoA oxidase. **COI1**, coronatine-insensitive1.

A putative receptor for systemin was isolated using a radiolabeled photo-affinity systemin that bounds to a protein of 160 kDa, the systemin receptor 160 (SR160) turned out to be a brassinosteroid receptor (BRI1) homologue (Scheer and Ryan, 2002). To verify that SR160 was the systemin receptor, the *cu3* null mutants which are non-functional in brassinolide (BL) signalling due to a mutation in the BRI1 gene, were assayed. Even if *cu3* mutants were expected to be insensitive to systemin, they showed an absence of PIs in response to systemin in some parts of the plant, while in other parts the response was significant (Scheer *et al*., 2003). In addition, Holton and others (2007) tested the same mutants and they demonstrated that *cu3* plants are not defective in systemin-induced wound signalling. Recently, the BRI1 receptor was found to be unnecessary for systemin signalling in tomato and not sufficient to give systemin sensitivity in tobacco leaves (Malinowsky *et al*., 2008).
Furthermore, prosystemin was also synthesized with an *Escherichia coli* and baculovirus/insect cell expression systems (Delano *et al*., 1999; Vetsch *et al*., 2000); the synthetic prosystemin was active when applied to wound sites in tomato leaves.
Also, mutants of systemin peptide were also assayed (Pearce *et al*., 1993). The substitution-deletion experiments suggested that the residues close to the N-terminal may be essential for interacting with a putative receptor, and the residues close to the C-terminal are required for activity. Tomato plants and *Lycopersicon peruvianum* suspension-cultured cells were assayed with synthetic prosystemin, systemin and mutated peptides to determine the bioactivity (Dombrowski *et al*., 1999), they showed that the absence of the systemin sequence in the prosystemin gene completely eliminate its proteinase inhibitor induction, indicating that the systemin sequence has

to be present for signal activation. Nevertheless, to date the role of the prosystemin lacking the systemin sequence has only been studied *in vitro.*
The tomato prosystemin cDNA was also expressed in potato (*Solanum tuberosum*) in its sense orientation (Narváez-Vásquez and Ryan, 2002). Transgenic plants showed high levels of proteinase inhibitors in leaves and tubers, similar to the effect of the over-expression of prosystemin in tomato. Prosystemin has been found in some species of the Solanaceae family and it is not found in other plant families. Prosystemin homologues have been isolated from potato, bell pepper and black nightshade (Constabel *et al*., 1998) all members of the *Solaneae* subtribe. The different prosystemins display high amino acid sequence identity when compared with tomato prosystemin. Identity varies from 73 to 88% (table 1). Two prosystemin genes were found in potato, the sequences of the two potato peptides were very similar with 96% identity.

Table 1. Amino acid identity of prosystemin proteins from tomato (TomProsys), potato (PotProsys1, PotProsys2), black nightshade (NigProsys) and bell pepper (Pep Prosys). Values are in percent.

	TomProsys	PotProsys1	PotProsys2	NigProsys
PotProsys1	88			
PotProsys2	86	96		
NigProsys	81	81	78	
PepProsys	73	76	74	73

The deduced systemin peptides from the three different species are very similar to the tomato systemin. They show only two or three amino acid substitutions in the N-terminal of the molecule and no substitution are present at the C-terminal region (table 2).

Table 2. Systemin amino acid sequences of four Solanaceae species

Prosystemin	Specie (common name)	Systemin peptide	
		* * ** ** *******	
TomProsys	*Solanum lycopersicum* (tomato)	1 AVQSKPPSKRDPPKMQTD	18
PotProsys1	*Solanum tuberosum* (potato)	AVHSTPPSKRDPPKMQTD	
PotProsys2	*Solanum tuberosum* (potato)	AAHSTPPSKRDPPKMQTD	
NigProsys	*Solanum nigrum* (black nightshade)	AVRSTPPPKRDPPKMQTD	
PepProsys	*Capsicum annum* (bell pepper)	AVHSTPPSKRPPPKMQTD	

Stars indicate identical residues

To find out if these systemin homologues also induced proteinase inhibitors, different synthetic systemins were applied to wound sites of tomato leaves (Constabel *et al*., 1998). Only the nightshade systemin demonstrate to be less active that the tomato one and systemins of potato and bell pepper were as active as tomato systemin. Afterwards, the diverse systemins were tested in their respective species, showing

proteinase inhibitor induction (Constabel *et al*., 1998). These experiments suggest that the prosystemins generate functional systemin peptides, as the various systemins induce defence genes in leaves after wounding.
Prosystemin homologues have not been identified in tobacco (*Nicotiana tabacum*), another member of the Solanaceae family, included in the *Nicotianae* subtribe. However, tobacco exhibits a wound response similar to tomato, as for instance wounding systemically activates the synthesis of trypsin inhibitors.
Even if Pearce and others (2001) showed that the tomato systemin did not provoke an alkalinisation of the tobacco cell-culture medium when supplied to tobacco plants through their cut stems, and that the synthesis of tobacco trypsin inhibitor in leaves was not activated, recently studies have shown conflicting results. A proteomic approach in tobacco plants transformed with the tomato prosystemin cDNA showed a significant accumulation in amount and number of proteins in leaves; the identified proteins were involved in oxidative stress, environmental stress and defence against pathogens (Rocco *et al.* 2008). This was confirmed later by Malinowski and others (2009), they showed that calli and suspension cultured cells from untransformed tobacco responded to systemin by showing both mitogen-activated protein kinase (MAPK) activation and a weak medium alkalinisation.

3.3. Systemin-like peptides

On the basis of the alkalinisation assay used to isolate the tomato systemin (Pearce *et al*, 2001), it was possible to isolate two peptides from tobacco leaves, with sequence peptides diverse to tomato systemin and with similar function, they were called systemin-like peptides. Hydroxyproline-rich systemins (HypSys) are small defence signalling glycopeptides isolated within the Solanaceae family that induce defence genes after wounding or herbivore attack. They were called systemin-like peptides as they were polypeptide defence signals that are produced by the plant in response to physical damage and that activate defence genes, either locally or systemically (Ryan and Pearce, 2003). HypSys glycopeptides were found in tobacco (*N. tabacum*), tomato (*S. lycopersicum*), blacknightshade (*S. nigrum*), potato (*S. tuberosum*), petunia (*Petunia hybrid*) and poplar (*Populus trichocarpa*) (Pearce *et al*., 2001; Pearce and Ryan, 2003; Pearce *et al*., 2007; Pearce *et al*., 2009). Very recently, a HypSys glycopeptide was isolated from sweet potato (*Ipomoea batatas*) of the Convolvulaceae family and found to induce the defensive gene *sporamin* (Chen *et al*., 2008). It is likely that HypSys defence peptides are no exclusive of the Solanaceae family.
Two HypSys glycopeptides were first isolated from tobacco and they are the best characterised. The two 18 amino acid glycopeptides contain multiple hydroxyproline residues (Pearce *et al*., 2001). Both peptides induce the synthesis of tobacco trypsin inhibitors when they are supplied to excised tobacco leaves through their cut stems. Also, TobHypSys I and II activate a MAPK with relative molecular mass 48K in tobacco suspension-cultured cells, similar to the 48K MAPK activation by tomato systemin in tomato cells (Stratmann and Ryan, 1997).
The two tobacco HypSys and the tomato systemin have a common characteristic, the presence of proline (P) or hydroxiproline (O) residues. TobHypSys I and II are not similar to each other nor to tomato systemin. The comparison of the amino acid sequences of both TobHypSys I and II is showed in table 3.

Table 3. Amino acid sequences of the tobacco hydroxyproline-rich glycopeptides, TobHypSys I and II compared with the tomato systemin peptide

Peptide	Amino acid sequence
Tomato Systemin	[1] AVQSKPPSKRDPPKMQTD [18]
TobHypSys I	RGANLPOOSOASSOOSKE
TobHypSys II	NRKPLSOOSOKPADGQRP

The HypSys I and II derive from a single 165 amino acid precursor, preproTobHypSys-A. HypSys I resides near the N-terminal region and HypSys II near of the C-terminal region. This was the first report in plants of two peptide signals being derived from a single precursor, a scenario common to animal and yeast. The TobHypSys-A precursor, from which TobHypSys I and II are released, has been reported to be induced by mechanical and herbivorous wounding systemically (Rocha-Granados *et al*., 2005). Furthermore, transgenic plants over-expressing the TobHypSys-A precursor increased the resistance against *Helicoverpa armigera* larvae in tobacco by inducing PIs and PPO activity (Ren and Lu, 2006).
In tomato three HypSys peptides were isolated from leaves. They have similar characteristics to the TobHypSys peptides, as they are small hydroxyproline-rich glycopeptides between 15 and 20 amino acids and they derive from a single precursor protein, a peptide of 146 amino acids (Pearce and Ryan, 2003). It was also showed that the TomHypSys precursor is wound-inducible in tomato leaves, as the tomato prosystemin (Pearce and Ryan, 2003). The two precursor peptides are represented and compared with tomato prosystemin (Fig. 3).

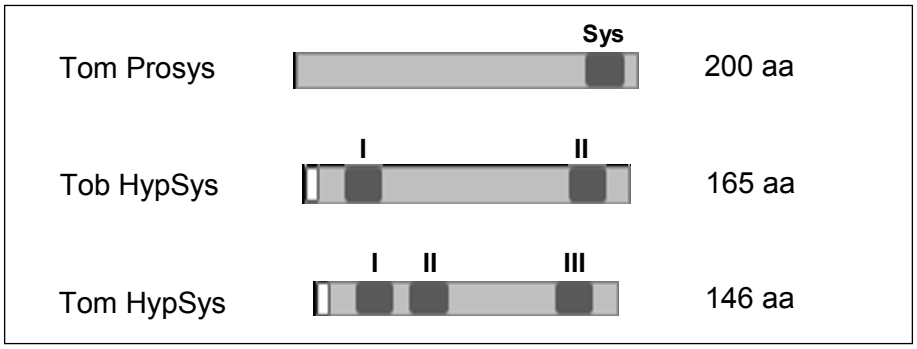


Fig. 3. Schematic representation of the tomato and tobacco systemin peptides precursors. White boxes represent the signal sequences. The Sys and Hypsys peptides are in represented as black boxes. Length of each precursor is shown on the right (Ryan and Pearce, 2003).

Even if the three tomato HypSys peptides are potent inducers of defence genes when applied to excised tomato plants, they are not a primary signal, this was demonstrated before by Orozco-Cárdenas and others (1993) as they studied tomato plants transformed with the antisense prosystemin gene, the transgenic plants were not able to generate a systemic signalling after wounding. Analysis of transgenic tomato plants over-expressing sense and antisense constructs of the tomato HypSys precursor showed that the expression of both the tomato HypSys precursor gene and the prosystemin gene in response to wounding are necessary for strong systemic signaling (Narvaez *et al*, 2007).

3.4. The octadecanoid pathway in Solanaceae

After the releasing of systemin by proteolitic cleavage of prosystemin, a complex wound cascade begins in the plasma membrane of tomato plants. The wound cascade ends with the activation of a phospholipase A_2 (PLA_2) which releases linolenic acid (18:3) from the plasma membrane, providing the substrate for the first step of the octadecanoid pathway (Ryan, 2000). The pathway from linolenic acid to jasmonic acid was called the octadecanoid pathway (Vick and Zimmerman, 1984). The octadecanoid pathway is one of the best characterised biosynthetic pathway for the induction of defence genes through the production of jasmonic acid, in several plant species. This molecule and its relative compounds (jasmonates) function as essential mediators in plant response to wounding, herbivore and pathogens. The jasmonic acid-dependent wound-signal transduction pathway of the tomato is represented in fig 4.

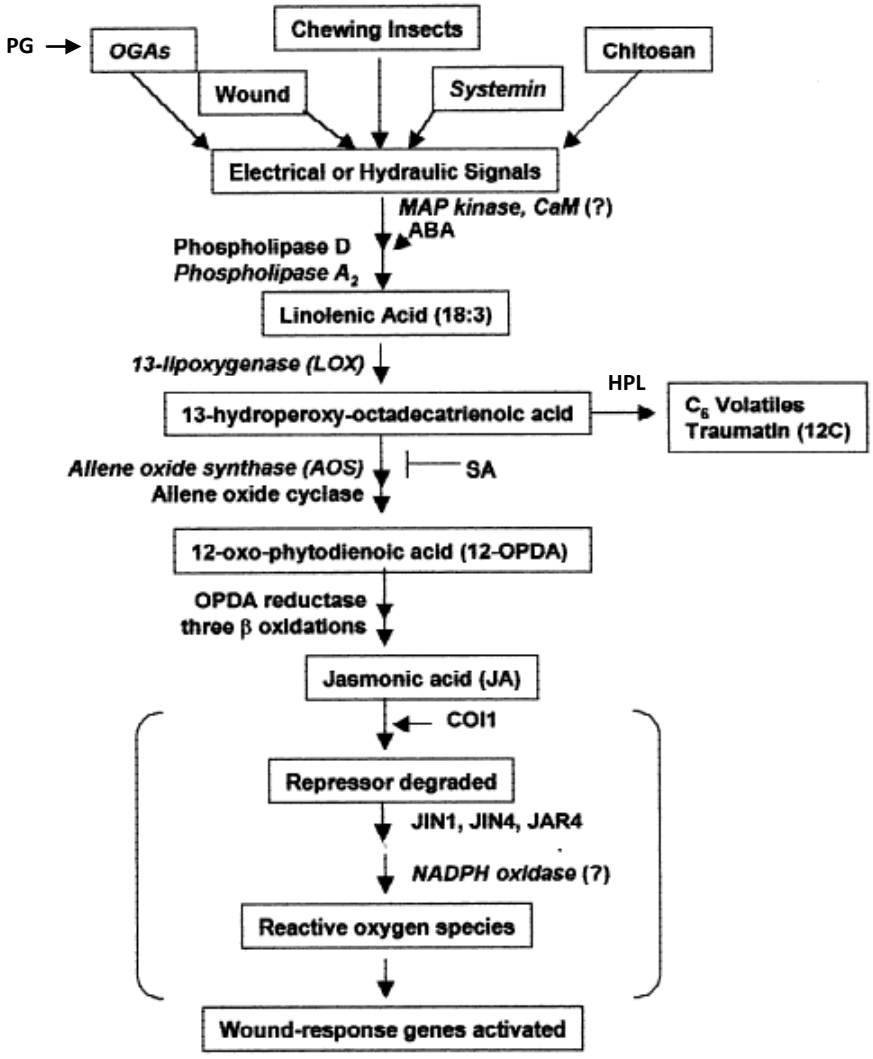


Fig. 4. Wound signal transduction pathway in tomato. The signal pathway is induced by herbivores, mechanical damaged, systemin, chitosan and OGAs. Systemin peptide is generated and transported in the course of the phloem. Phospholipases liberate linolenic acid from membranes that it is converted in 13-hydroperoxide by LOX. The 13-hydroperoxide has two possible destinies, to generates C_6 volatiles or proceeding into the octadecanoid pathway to generates jasmonic acid, a key signal for the activation of defence-related genes. The area in brackets is speculative. Modified from Walling, 2000.

The octadecanoid pathway is induced by mechanical wounding and herbivores in Solanaceous. Insects feed on plants not only provokes mechanical damage but insects also release salivary secretions that might introduce elicitors. Elicitor are any compound that comes from herbivores and interacts with the plant at a cellular level (Kessler and Baldwin, 2002). A wound-induced polygalacturonase (PG) hydrolyzes pectin in the cell wall to release oligogalacturonides (OGAs), which are potent signals that activate the tomato octadecanoid pathway (Walling, 2000; Bergey *et al*., 1999). Mechanical wounding also generates electrical or hydraulic signals that are quickly propagated from the wound site (Rhodes *et al*., 1999). These signals might be involved in the amplification of the signal cascade all over the plant. In addition, the signal peptide systemin is produced and transported through the phloem to mediate both local and systemic activation of the octadecanoid pathway (Ryan 2000). OGAs, chitosan, and systemin cause the raise of in cytosolic calcium, MAPK activity, inactivation of H+ ATPase, membrane depolarization, generation of reactive oxygen species (ROS), PLA_2, etc (Schaller, 1999). Then, phospholipases release linolenic acid from membranes (Narváez-Vásquez *et al*., 1999). Afterwards, lipoxygenases convert linolenic acid to a 13-hydroperoxide, which could go through two different ways. First, it may be hydrolyzed by hydroperoxide lyase (HPL) to generate C_6 volatiles and traumatin, or may continues the octadecanoid pathway. 13-hydroperoxide interacts with the allene oxide synthase (AOS) and allene oxide cyclase (AOC) enzymes to generate 12-oxo-phytodienoic acid (12-OPDA). Finally, consecutive β-oxidation steps generate jasmonic acid and also methyl jasmonic acid, key signals for the transcriptional activation of defence-related genes (Stenzel *et al*., 2003; Walling, 2000; Schaller, 1999; Vick and Zimmerman, 1984).

3.5. Research objectives

The aim of this project was to increase the understanding of the possible biotechnological role of the tomato prosystemin as enhancer of the endogenous resistance level against biotic stress in other Solanaceae. This aim was pursued by studying the expression of tomato prosystemin in tobacco and potato. Besides, a mutated prosystemin cDNA lacking the 3' terminal systemin encoding exon was also expressed in both Solanaceae to verify the possible function of the N-terminal region prosystemin precursor in the activation of the defence response. The schematic representation of the cassettes used for plant transformation are shown in fig. 5.

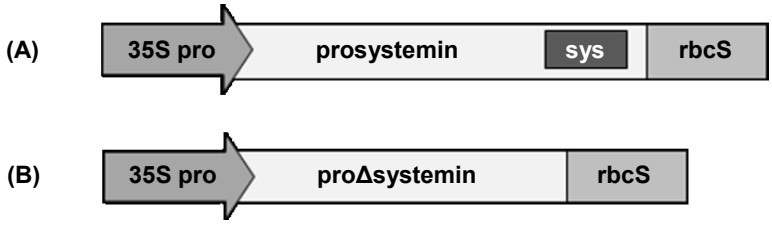


Fig. 5. Expression cassettes used for plant transformation. **(A):** cassette with the prosystemin cDNA to produce MZ plants. **(B):** cassette with the deleted prosystemin to produce PRO8 plants. **35S pro**: 35S RNA CaMV promoter; **prosystemin:** cDNA of the tomato prosystemin gene; **rbcS:** ribulose-1,5-bisphosphate carboxylase oxygenase small (RuBisCoS) terminator sequence; **sys:** systemin coding sequence; **proΔsystemin:** cDNA of the tomato prosystemin gene lacking the 11th exon coding the systemin peptide.

4. MATERIALS AND METHODS

4.1. Genomic DNA extraction

Putative transformants that developed roots in the selective medium were collected in liquid nitrogen and conserved at -80°C until processing. Approximately, 100 mg of leaf tissue was frozen and ground in liquid nitrogen. Genomic DNA was isolated using the Gen Elute Plant Genomic DNA miniprep kit (Sigma-Aldrich). Subsequently, the genomic DNA was quantified by electrophoresis in 1% agarose gel stained with ethidium bromide, by comparison to a Lambda DNA (Promega) of known quantity. The genomic DNA was stored at -20°C.

4.1.1. Polymerase Chain Reaction (PCR)

For PCR amplification, a 50 µl of master mix was prepared containing 25-100 ng of DNA template, 0.4 µM primers, 1.5 mM $MgCl_2$, 100 µM dNTPs and 1 U Taq DNA Polymerase (Promega) in 1X PCR buffer (Promega). The specific primers reported in table 4 were used for the prosystemin gene amplification in tobacco and potato transgenic plants. The PCR reaction was performed in a Mastercycler Gradient (Eppendorf) with a thermal cycling as showed in table 4. Amplification products were visualised in 2% agarose gel stained with ethidium bromide and UV light visualization.

4.1.2. DNA sequencing

All the amplification product (50 µl) was loaded into 0.8% agarose gel with a 1Kb plus DNA Ladder (Invitrogen) to control the amplicon length. The target bands were cut to obtain a maximum of 0.4 g of gel. Afterwards, the DNA was isolated from the agarose gel with the QIAquick Gel Extraction kit (QIAGEN) to obtain 50 µl of DNA solution. DNA was quantified in 0.8% agarose gel by comparing with a known concentration of Lambda DNA (Promega). The primers and the DNA were dissolved in water and they were sending at the concentration and quantity requested for the DNA sequencing service PRIMM (Naples). The sequencing was performed in the 3730 DNA Analyzer (ABI) Sequencer. The results were analysed and aligned with Clustal W2 software program (http://www.ebi.ac.uk/Tools/clustalw2/).

4.2. RNA analysis

4.2.1. Total RNA extraction

Total RNA was extracted from leaves using a standard method based on a phenol/chloroform extraction. Leaves were harvested and frozen in liquid nitrogen, ~ 0.5 g of plant material was ground and added to a 2-ml-tubes containing 750 µl of extraction buffer (100 mM Tris-HCl pH8.5, 100 mM NaCl, 20 mM EDTA, 1% SDS) and 750 µl of phenol-chloroform (1:1). After mixing in a vortex, the tubes were centrifuged. All the centrifugation steps were performed at 14000 rpm for 10 min at 4°C. The supernatant was collected and phenol-chloroform extraction was repeated. After centrifugation the supernatant was transferred to a tube containing 750 µl of chloroform and centrifuged as above; 750 µl of cold isopropanol were added to the

supernatant and mixed. The pellet was collected by centrifugation and after isopropanol evaporation, pellets were resuspended in 200 µl of bi-distilled water. RNA was precipitated by adding 1 volume of 4M LiCl and stored at 4°C for at least 4 hours. RNA pellet was collected by centrifugation for 15 minutes. The pellet was re-suspend in 400 µl of water, then 40 µl of NaAc 3M (pH 7.0) and 1 ml of 96% ethanol were added. Afterwards, it was cooled at -80°C for 10 minutes and the pellet was recuperated by centrifugation. Total RNA was resuspended in 42 µl of water.

4.2.2. Control of the RNA

Total RNA was quantified with a spectrophotometer (Eppendorf Biophotometer), 2 µl of total RNA were diluted 1:100 and the nucleic acid concentration was measured at 260 nm. The purity of total RNA was verify with the A_{260}/A_{280} ratio. Then, RNA quality was controlled by electrophoresis in 1% agarose gel in a denaturing loading buffer (400 µl formamide, 120 µl formaldehyde, 80 µl 1% TAE, 1.2 µl ethidium bromide, 20 µl 6X Loading Dye). The loading buffer together with 4 µg of total RNA were incubated at 65°C for 2 minutes before electrophoresis. Total RNA was visualised in 1.2 % agarose gel.

4.2.3. Reverse transcription

Ten µg of total RNA were treated with RNase-free DNase I (BioLabs) to eliminate any DNA contaminant (1U DNase/3 µg RNA) in 1X NEBuffer for DNase I (BioLabs) and it was incubated at 37°C for 20 minutes. RNA was precipitated by adding 1/10 volume of NaAc 3M (pH 7.0), 2 volumes of 100 % ethanol, cooled at -80°C for 10 minutes, and centrifuged at 14000 rpm for 15 minutes at 4°C. The pellet was washed with 1 volume of 70 % ethanol and cooled again at -80°C for 10 minutes, centrifuged at 14000 rpm for 10 minutes and then resuspended in 20 µl water. For the mRNA reverse transcription was used the RevertAid First Strand cDNA synthesis kit (Fermentas), following the manufacturer's instructions. First strand cDNA was synthesised from 2 µg of total RNA (DNA free) and the cDNA template was conserved at -20°C until analysing.

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4.2.4. RT-PCR

The cDNA synthesis was checked by using the Elongation Factor-1 alpha (EF-1α) primers for *Solanum tuberosum*, and *Nicotiana tabacum*. Primers were designed to anneal on two consecutive exons to detect any DNA contaminant. The specific primers reported in table 4, were used for the cDNA synthesis control, and for the prosystemin gene amplification to control the transcription of the transgenes. The PCR reaction was prepared in 25 µl containing 1 µl of cDNA template, 0.4 µM primers, 1.5 mM $MgCl_2$, 100 µM dNTPs and 0.5 U Taq DNA Polymerase (Promega) in 1X PCR buffer (Promega). The thermal cycling was performed as shown in table 4, with thirty cycles of amplification in a Mastercycler Gradient (Eppendorf). The amplification products were controlled in 2% agarose gel electrophoresis stained with ethidium bromide.

Table 4. Primers for the amplification of the EF1-α and prosystemin genes.

PRIMERS	SEQUENCE (5' – 3')	GENE (SPECIE)	ACC. NUM. (REFERENCE)	THERMAL CYCLING	A.L. (bp)
Prosys fw Prosys rv	GGGAGGGTGCACTAGAAATAA TTGCATTTTGGGAGGATCACG	TomProsys *S. lycopersicum*	M84801 (McGurl *et al.*,1992)	94°C 30'' 51°C 30'' 72°C 2'30''	110
EF1 St fw EF1 St rv	AAGCTGCTGAGATGAACAAG CAGTGTCAACACGACCAAC	EF 1-α *S.tuberosum*	AB061263 (Nakane *et al.*, 2003)	94°C 45'' 53°C 45'' 72°C 45''	639
NT-EF fw NT-EF rv	AGACCACCAAGTACTACTGC CTCTTCTTGAGGCTCTTGAC	EF 1-α *N. tabacum*	D63396 (Kumagai *et al.* 1995)	94°C 45'' 55°C 45'' 72°C 45''	422
SYSdel fw SYSdel rv	GAAGGAGATGATGATGCACAA CACCCTCCCCTTCCATCT	TomProsys *S. lycopersicum*	M84801 (McGurl *et al.* 1992)	94°C 45'' 60°C 45'' 72°C 30''	208

ACC. NUM. = accession number; A.L.= amplicon length

4.3. Gene Expression analysis

The Real Time RT-PCR for the relative gene expression analysis was performed in the ABI PRISM 7000 Sequence Detection System (Applied Biosystem) and in the 7900 Fast Real-Time PCR System (Applied Biosystem) with a total volume of 25 µl containing 12,5 µl 2X of Power Sybr Green PCR Master Kit (Applied Biosystem) or 2X Quantifast Sybr Green PCR Kit (QIAGEN). The mix reaction included 0,3 µl of first strand cDNA template (section 4.2.3.) and 0,35 µM of specific primers. The thermal cycling program begin with 2 minutes at 50°C, 4 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at the Tm value of the primers showed in table 5. Three biological replicates were screened for every genotype. The amplification was also done with 3 repetitions for every amplification event. A step of dissociation was included to control the amplification products with 15 seconds at 95°C, 15 seconds at 58°C and slow denaturation at 95°C for 15 seconds, the melting curves obtained with this step were compared with the melting temperatures (Tm) of every pair of primers showed in table 5. The products were also controlled by electrophoresis in 2% agarose gel stained with ethidium bromide, the amplicon length of all the primers are reported in table 5. The output data was analyzed with the 7000 System Sequence Detection Software or the 7900HT Sequence Detection Systems Software, version 2.3 (Applied Biosystem), using the Comparative Ct method with the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001), where

$$\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{endogenous control}} \qquad \Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}$$

Elongation Factor 1-alpha gene for *S. tuberosum* and *N. tabacum* were the endogenous reference genes used to normalize the expression levels of the target genes in potato (Nicot *et al*., 2005) and in tobacco (Rotenberg *et al.,* 2006).

4.3.1. Primers Design

The gene specific primers reported in table 5 were used for the gene expression analysis of tobacco and potato transgenic plants. The primers were designed with Primer Express 2.0 Software (Applied Biosystems). The fragment to be amplified was chosen between 80 to 150 bp.

Table 5. Gene specific primers used for gene expression analysis by Real Time PCR in tobacco and potato.

PRIMERS	SEQUENCE (5' – 3')	GENE	ACCESSION NUMBER	REFERENCE	Ta °C	A.L. (bp)
NtCaM fw NtCaM rv	TCACTTGGTCAGAATCCCACT TCCACCTCTTCATCAGTCAGC	Calmodulin 1	AB050837	Yamakawa *et al*., 2001	58	254
NtGST fw NtGST rv	AATTTGAACCCCCGGCTACA TTGCGCTTCGCTTTCCTTC	Glutathione S-transferase	D10524	Takahashi and Nagata, 1992	58	101
NtHSP fw NtHSP rv	TCTTCGATCCATTCTCCCTCAA AGCAGCGGTTTCACGAGTAGAG	Low molecular weight heat-shock protein	AF166277	Park and Hong, 1998	58	101
Nt EF fw Nt EF rv	TCTGTTGAGATGCACCACGAAG ACAAACCCACGCTTGAGATCC	Elongation factor 1-alpha	D63396	Kumagai *et al.* 1995	58	104
NtPin II fw NtPin II rv	TGTAACGCAGACAAGGGTTGC AAGCAGCACTTTGAGGCTCC	Proteinase inhibitor II	Z29537	Balandin *et al*., 1995	58	82
NtRBP fw NtRBP rv	TGAATACAGGTGCTTCGTCGG CGAGTCGAGAATTTCGCCATAC	RNA-binding protein	AF005359	Naqvi *et al.,* 1998	58	94
NtSAP fw NtSAP rv	AAACTGAGCAACCTTGGAGCC CGGTTGAGTATTTTGCTGCCA	Stromal ascorbate peroxidase	AB022274	Yoshimura *et al.,* 2000	58	109
NtTobsys fw NtTobsys rv	AGCTTCATCTCCACCGACC TTAATGTTTTCCCCTTGAGCAA	Hydroxyproline-rich glycopeptides I and II	AY033148-9	Pearce *et al.,* 2001	58	120
NtLox1 fw NtLox1 rv	TCAATTTGGCGCTGTTAGCA CAGTGAATGATTCGGGCGTT	Lipoxygenase 1	X84040	Veronesi *et al*., 1995	58	106
PotCaM fw PotCaM rv	TCATGACAAACCTAGGCGAAAA CGTCGTAGTTGATCTGCCCAT	Calmodulin	U20295	Takezawa *et al.,* 1995	58	96
PotEF fw PotEF rv	TTGGAAACGGATATGCTCCAG CCTTACCTGAACGCCTGTCAA	Elongation Factor 1- alpha	AB061263	Nakane *et al*., 2003	58	98
PotGluB2 fw PotGluB2 rv	TGCATTTGGTGCCACACAA AGGTCCAGGCTTTCTCGGA	Endo-1,3-beta-D-glucanase	U01901	Beerhues and Kombrink, 1994	58	91
PotHPL fw PotHPL rv	CGGCGTAAATCCAGAAAACTT ACTTGGCACTGGTGGAGTAAGC	Hydroperoxide lyase	AJ310520	Vancanneyt *et al*., 2001	58	102
PotLox3 fw PotLox3 rv	GTCGAAGATTCATGCCTGAGC TGTCTGCAGTTGAGGAGTGATTGT	Lipoxygenase 3	U60202	Kolomiets *et al.,* 1996	58	101
PotPR-1b fw PotPR-1b rv	CTGGCGCAACTCAGTTCGA GTCCGACCCAGTTTCCAACA	Pathogenesis-related protein 1b	AY050221	Hoegen *et al*., 2001	58	101

A.L.=amplicon length. Ta= annealing temperature

PRIMERS	SEQUENCE (5' – 3')	GENE	ACCESSION NUMBER	REFERENCE	A.L. (bp)	Ta °C
PotProHypSys fw PotProHypSys rv	GCCAGAGCCACAAGATGAGC GGGCTGAGGTCCACTTCAAC	Hydroxyproline-rich glycopeptide	EU482409	Bhattacharya *et al*., 2008	58	101
PotProsysfw PotProsys rv	AGGTGGTATGTGAGGAAAGAG TTATTAGATTTTGGATATTCTAGAG	Potato prosystemin 1 and 2	AF000373/4	Constabel *et al.,* 1998	53	135

A.L.=amplicon length. Ta= annealing temperature

4.4. Proteins extraction and analysis

4.4.1. Total protein extraction

Leaves were frozen in liquid nitrogen, and 0.5 g of vegetal tissue were ground to a fine powder. A liter of 10X phosphate buffered saline (PBS) was prepared with 87 g NaCl, 22.5 g Na_2HPO_4, 2 g KH_2PO_4, at pH 7.4 (Sambrook and Russell, 2001). Proteins were extracted in 300 µl of PBS buffer diluted 1:10, containing 0.5 M NaCl, 10 mM EDTA, 1 mM PMSF, the suspension was mixed vigorously with a vortex for 3 minutes until homogenization. The samples were centrifuged at 13000 rpm for 20 minutes at 4°C, the supernatant was collected. Total soluble proteins were quantified by spectrophotometry (Eppendorf Biophotometer) with the Bradford method, using the bovine serum albumin (BSA) as a protein standard (Bradford, 1976).

4.4.2. SDS-PAGE

Total soluble proteins were separated in a 12% SDS Polyacrylamide Gel Electrophoresis (PAGE), Resolving gels at 12% were prepared with 8 ml of 30% acrylamide, 5 ml of 1.5 M Tris pH 8.8, 0.2 of 10% SDS, 0.2 of 10% ammonium persulfate, 8 µl of TEMED and water until 20 ml. The 5% stacking gels were prepared with 670 µl of 30% acrylamide, 500 µl of 1.0 M Tris pH 6.8, 40 µl of 10% SDS, 40 µl of 10% ammonium persulfate, 4 µl of TEMED in 4 ml.

A volume of 15 µl containing 40 µg of total soluble proteins was mixed with an equal volume of 2X sample buffer (0.8 ml of 0.5 M tris-HCl pH 6.8, 2 ml glycerol, 0.2 ml of 0.01% blue bromophenol, final volume of 10 ml). The samples were heated at 100 °C for 5 minutes before loading. A 5X Tris-Glycine-SDS solution was prepared (94 g Glycine, 15.1 g Tris, 50ml 10% SDS for 1 litre, pH 8.8). It was diluted 1:5 to obtain the 1x running buffer. The electrophoretic run was performed in the Mini Protean Tetra Cell (Bio Rad) at 100 volts for ~1 hour. The Precision Plus Protein standard (Bio Rad) was used to determinate molecular weights.

4.4.3. Electroblotting

After electrophoresis, two identical gels were prepared for the Western analysis. A gel was stained with Coomassie blue to control the quality and quantity of proteins. The other gel was electroblotted to nitrocellulose membranes (Bio Rad) for inmunoblotting. The protein transference was carried out in the Trans-Blot Tranfer Medium (Bio Rad) in 1X electroblotting buffer (50 mM Trizma, 380 mM Glycine, 10% methanol) at 100 volts for 1 hour.

4.4.4. Western blot

Non-specific sites were blocking by soaking the nitrocellulose membrane in a blocking solution of 5% free-fat milk powder and 0.5% Tween20 in 1x PBS buffer for at least 1 hour. Every incubation and wash step was done with gently rotation at room temperature. The membrane was incubated with the primary antibody (anti-prosystemin) kindly provided by Prof A. Schaller from the University of Hohenheim (Delano *et al*., 1999), the anti-prosystemin was diluted 1:1000 in 1X PBS with 5% free-fat milk powder and 0.5% Tween20 for 3 hours. Membranes were washed 4

times for 10 minutes in 1X PBS with 0.5% Tween20. After, the secondary antibody (peroxidase labelled anti-rabbit, IgG, Amersham) diluted 1:10000 was added in 1X PBS with 5% free-fat milk powder and 0.5% Tween20 for 1 hour. Membranes were washed again 4 times for 10 minutes in 1X PBS with 0.5% Tween20 and once with 1X PBS for 10 minutes. The prosystemin proteins were visualized at 30 minutes of exposition time, with the ECL Western Blotting Detection System (Amersham) as recommended by the manufacturer.

4.5. Plasmid DNA extraction from *Escherichia coli*

The binary vectors pMZ (prosystemin cDNA) and pPRO8 (deleted prosystemin) were already available in *Escherichia coli* DH5α strain at the Prof Rao's Molecular Biology Laboratory. The pMZ and pPRO8 maps are showed in fig. 6.

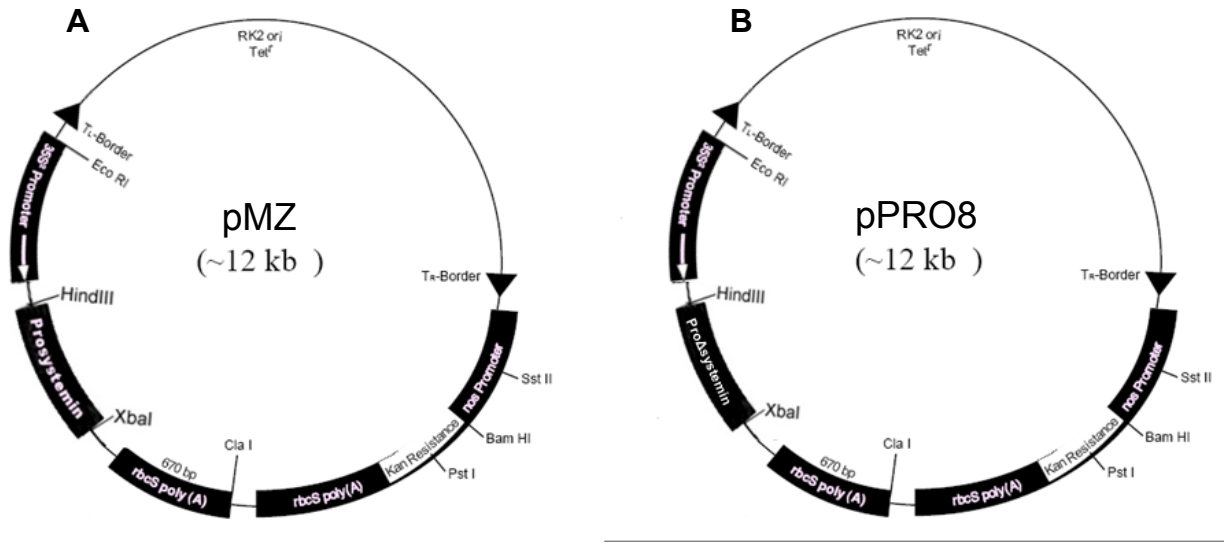

Fig. 6. Map of the pMZ and pPRO8 plasmids used for plant transformation. **A:** The binary vector pMZ, containing the expression cassette with the kanamycin resistance gene and the expression cassette with the tomato prosystemin cDNA under the control of the 35S cauliflower mosaic virus promoter and the rbcS terminator, TL and TR are the left and right T-DNA borders, respectively. **B:** Same binary vector containing the deleted tomato prosystemin.

The bacteria conserved at -80°C was inoculated in LB medium (Sambrook and Russell, 2001) solidified with 15 g/l of bacteriological agar and supplemented with 50 µg/ml tetracycline. After inoculation, the plates were incubated overnight at 37°C. A single colony of transformed bacteria was picked up and inoculated in a 100-ml flask containing LB broth with 50 µg/ml tetracycline. The flasks were incubated overnight for 16 hours at 37°C with 200 rpm shaking.
Plasmid DNA was isolated with the NucleoBond Midi Plasmid DNA purification (Macherey-Nagel). Plasmid DNA integrity and yield were checked by electrophoresis in 1% agarose gel stained with ethidium bromide in 1% TAE running buffer. Plasmid DNA was conserved at -20 °C.

4.6. Plant genetic transformation

4.6.1. Agrobacterium transformation

The plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 strain. Chemically competent cells were obtained by using a chemical method. A flask with 5 ml LB broth (prepared as in section 4.5) was inoculated with *A. tumefaciens* and then incubated overnight at 28°C, with 200 rpm shaking. After 20 hours incubation, 2 ml of the bacterial suspension was added to a 50 ml YEP broth (10 g peptone, 10 g yeast extract, 5 g NaCl for 1 litre medium). The bacteria culture was placed on a growth chamber at 28°C with 200 rpm shaking for 8 hours until the suspension reached an OD_{600} ~1.0. The bacterial cells were then collected by centrifugation at 3000 rpm, for 5 min at 4°C and resuspended in 1 ml $CaCl_2$ 20 mM. The *Agrobacterium* competent cells were divided in 1-ml aliquots and stored at –80°C until transformation.
For *Agrobaterium* transformation, 1 µg of plasmid DNA was added to every tube containing the *Agrobacterium* competent cells. Cells were flash frozen in liquid nitrogen for 5 seconds, and then immediately incubated in a water bath at 37°C for 5 minutes. Subsequently, 1 ml LB broth was added to every tube. After incubation at 28°C with rotary shaking for 2 hours, the bacterial cells were collected by centrifugation at 3000 rpm for 5 minutes and the pellet was resuspended in 0.2 ml LB. The putative transformed cells were inoculated in LB agar with the appropriate antibiotics (tetracycline 5 µg/ml and rifampicin 50 µg/ml). Colonies were grown at 28°C for 3 days on solid LB.

4.6.2. Plasmid DNA extraction from *Agrobacterium tumefaciens*

A single colony of transformed *A. tumefaciens* cells was used to inoculated 10 ml LB broth with antibiotics (tetracycline 5 µg/ml and rifampicin 50 µg/ml). The flasks were incubated at 28°C with 20 rpm shaking for 24 hours. A volume of 2 ml of every flask was conserved in tubes with sterile glycerol (300 µl of cell suspension in 600 µl of 50% glycerol) and placed at -80°C. The remaining 8 ml of cell suspension of every flask were used for plasmid DNA extraction using a mini-prep protocol.
The pellet of the cells was obtained by centrifugation at 4300 rpm for 10 minutes. It was then resuspended in 533 µl of cold Solution I (50 mM glucose, 25 mM TrisCl, 10 mM EDTA, pH 8.0). Tubes were placed on ice for 10 minutes. A fresh Solution II was prepared (0.2M NaOH and 1% SDS), 1100 µl of Solution II was added to the flask, then it was mixed by inverting and cooled on ice for 5 minutes. Afterwards, it was mixed gently with 800 µl of Solution III (60 ml 5M potassium acetate**,** 11.5 ml glacial acetic acid**,** 28.5 ml bi-distilled water) and placed on ice for 5 minutes. The solution was transferred to a 2-ml tube to be centrifuged. All the centrifugation steps were performed at 13200 rpm for 5 minutes at 4°C. The supernatant was collected, 500 µl of phenol-chloroform (1:1) was added and then centrifuged as before. The supernatant was collected again, 10 µl RNAse (10 mg/ml) was added and incubated at 37°C for 30 minutes. Then, 500 µl of phenol-chloroform were added and the supernatant was collected in a new tube by centrifugation. The supernatant was recuperated again after adding 500 µl of chloroform and a centrifugation step. DNA was precipitated with the addition of 1 ml of 100% ethanol and incubation for 5 minutes at -80°C. The pellet was collected by centrifugation and washed with cold

70% ethanol, the pellet was recuperated by centrifugation and it was dried off. Finally, it was resuspended in 50 µl of bi-distilled water.
Plasmid DNA quality and quantity were controlled by electrophoresis in 1% agarose gel stained with ethidium bromide in 1% TAE running buffer. The plasmid DNA solution was stored at -20°C to be analysed by PCR.

4.6.3. Keto-lactose test

A. tumefaciens and *E. coli* cells containing pMZ and pPRO8 were inoculated on a solid medium of lactose agar containing 1% lactose , 0.1% yeast extract and 2% bacto-agar. Plates were incubated at 30°C for 3 days. Transformed *E. coli* cells were used as negative control. All the plates with the bacterial colonies were flooded with a shallow layer (8 ml) of Benedict's reagent prepared fresh with 132 g of sodium citrate, 256 g of sodium carbonate anhydrous ($Na_2CO_3.10H_2O$) and 13.5 g of copper sulfate ($CuSO_4.5\ H_2O$) per litre. Plates were incubated at room temperature for 30 minutes. A yellow ring of Cu_2O appeared wherever there was 3-ketolactose produced by *Agrobacterium*.

4.6.4. Plant material

Solanum tuberosum, cv. Desiree was grown *in vitro* in 100 ml MS20 medium (4.3 g MS basal salts including vitamins, 20 g sucrose for 1 litre medium, pH 5.8) in glass jars with slow rotary shaking for gas exchange, at 20°C to 22°C under a 16-hour photoperiod. The stem internodes were ready for genetic transformation at 4 weeks after culture.

4.6.5. Agrobacterium culture

Agrobacterium strain frozen stock (section 4.6.2) was inoculated on LB agar with selective antibiotics (tetracycline 5 µg/ml, rifampicin 50 µg/ml). Plates were incubated at 28°C for 2 days. A single colony was used to inoculate 20 ml of LB broth with selective antibiotics in a 50-ml flask. Cells were grown overnight at 28°C with ~200 rpm shaking for 1 day. The culture concentration was estimated by measuring absorbance at 600 nm until a density between 0.8 and 1.0, OD_{600}.
Agrobacterium virulence was induced by using acetosyringone in simplified induction medium (SIM) (20 mM sodium citrate, 2% sucrose, 0.3 mM acetosyringone). The 1-day bacterial culture was centrifuged at 3000 rpm for 3 min, the pellet was resuspended in SIM and incubated at 28°C overnight with rotary shaking. The bacterial culture was centrifuged at 3000 rpm for 3 min and the pellet was resuspended in 20 ml of LB broth with selective antibiotics (tetracycline 5 µg/ml, rifampicin 50 µg/ml) for co-cultivation.

4.6.6. Potato transformation

Potato plants were transformed as described by Narváez-Vásquez and Ryan (2002) with some modifications. Stem internodes explants of 4-week-old *in vitro* were clean cut (either 0.5-1.0 cm stem internodes), putting attention to eliminate any nodal region attached to the explants. Twenty explants per plate were placed in a disc of moist filter paper covering MS30 agar (4.3 g MS basal salts with vitamins, 30 g sucrose, 8 g micro agar for 1 litre, pH 5.8). They were incubated at 22°C and 16-hour

photoperiod, covered with four layers of filter paper to maintain low light conditions, for 24 hours.
Explants were placed in *Agrobacterium* cell suspension (section 4.6.5) for 10 minutes. The excess of bacteria suspension was removed with a filter paper, the internodes were placed into the same MS30 plates in direct contact with the medium, in a growth chamber at 22°C, 16-hour photoperiod for 3 days. To maintain low light conditions, the plates were covered with four layers of filter paper.
The explants were transferred to fresh P55 agar medium (for 1 litre medium 4.3 g MS basal salts with vitamins, 30 g sucrose, 8 g micro agar, 3 mg/l zeatine riboside, 3 mg/l giberellic acid, 0.1 mg/l indolacetic acid, pH 5.8), including 200 mg/l cefotaxime and transformant-selecting antibiotic (100 mg/l kanamicyn). Plates were cover with two layers of paper to maintain low light conditions and then incubated at 22°C and 16-hour photoperiod.
Every 15 days, the explants were transferred to fresh P55 agar medium with 100 mg/l kanamicyn and 200 mg/l cefotaxime. As soon as the shoots appeared, paper cover was removed. When the first well-differentiated shoots reached ~2 cm long, they were cut eliminating any callus tissue attached to it. The shoots were placed in MS30 agar until rooting, including kanamicyn (100 mg/l) to select transformants and cefotaxime (200 mg/l) to inhibit *Agrobacterium* growth.

4.6.7. Micropropagation of rooted shoots

Putative transformants that were able to develop roots in the selective medium (100 mg/l kanamycin) were propagated *in vitro* in MS30 agar including 50 mg/l kanamicyn to obtain at least 3 plants for every transformant. They were grown at 22°C and 16-hour photoperiod.

4.6.8. In vivo transfer

Regenerated *in vitro* plants were transferred to sterile soil, plants were covered with a transparent plastic capsule to avoid dehydration for the first week. They were grown in a growth chamber at 20°C to 23°C under a 16-hour photoperiod until tuber production.

4.7. Plant Growth

4.7.1. Tobacco

Seeds of transformed and non-transformed *Nicotiana tabacum* cv Samsun were germinated in MS30 agar plates prepared as in section 4.2.3, containing 100 µg/ml kanamycin to select positive transformants, they were grown at 25°C under a 16-hour photoperiod. When 4 leaf appeared, the plants were transferred to sterile soil and covered with a plastic capsule to prevent high transpiration for the first 5 days. All the plants used for the experiments were 4 to 5-week old.

4.7.2. Potato

Transformed and non-transformed potato plants (*Solanum tuberosum* cv Desiree) were grown vegetatively from tubers in a growth chamber at 23°C to 25°C under a

16-hours photoperiod. Plants 5 to 6-weeks old were used for all experiments. Transgenic lines used in the experiments were selected after tubers homogeneity.

4.8. Fungal resistance bioassay

Four plants for genotype were used for the bioassay. *Botrytis cinerea* was inoculated in Potato Dextrose Agar medium with 20 µl of 10^6 spores/ml and then incubated at 25°C for 10 days. The plates were flooded with 8 ml of sterile water to collect the spores. Spore concentration was determined microscopically in a Burker counting cell. Spore suspension (10^6 spores/ml) was prepared in a germination buffer (20 mM glucose, 20 mM KH_2PO_4). Ten µl were used to inoculate leaves between nerves (4 spots per leaf). The area of lesion was measured at 4, 6 and 8 days after inoculation. The bioassay was performed in a growth chamber at 25°C, 90 % relative humidity and with 16-hour photoperiod.

4.9. Statistical analysis

Data were analysed either by one-way ANOVA followed by a Tukey HSD post-hoc-test for multiple comparisons, or a *t*-test in case of two populations. All analyses were done using the software package SPSS version 16.0

5. RESULTS

5.1. Expression of the tomato prosystemin cDNA in tobacco

5.1.1. Molecular characterization of transgenic plants

Molecular characterization was done on 4-week old transgenic tobacco plants. These were the MZ119 line carrying the prosystemin cDNA and the PRO8-56 line carrying the deleted prosystemin lacking the exon coding for the systemin peptide (fig 6). These transgenic lines were selected after analysing the genomic DNA of several transformants by Southern blot to determine the transgene copy-number (fig. 7).

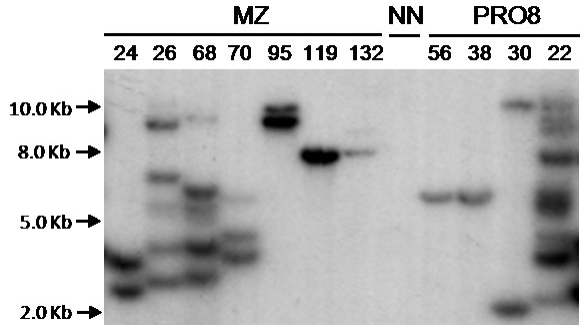

Fig. 7. Southern blot analysis of some transgenic lines. Numbers indicate independent transformants**. MZ:** plants carrying the prosystemin cDNA, **PRO8:** plants carrying the deleted prosystemin gene, **NN:** non-transformed plants.

Several inserts were visualised in some transgenic lines, except for MZ119, MZ132, PRO8-56 and PRO8-38 lines which showed a single insertion.
Total RNA was isolated from leaves of the transgenic lines (MZ119 and PRO8-56 lines). RNA integrity and yield was controlled in agarose gel electrophoresis. An example of electrophoresis of total RNA is shown in fig 8.

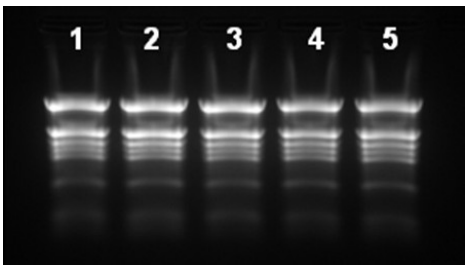

Fig 8. Agarose gel electrophoresis of total RNA from leaves (4µg/lane). **1-2:** MZ119 line, **3-4:** PRO8-56 line, **5:** non-transformed plant as control

The template cDNA quality was checked by RT-PCR with the primers for the Elongation Factor 1-alpha gene (EF 1-α). The primers (NT-EF forward and NT-EF reverse) were designed to anneal at the beginning of the transcribed sequence. The cDNA is 1671 bp long, and since the cDNA synthesis begins at the 3' terminal, their use can be a proof that the retro-transcription has produced filaments of 1400 nucleotides. Furthermore, NT-EF forward and NT-EF reverse were designed to anneal on two consecutive exons to verify the presence of possible contaminant genomic DNA. The amplicon length with a cDNA template is 422 bp, while a bigger

fragment is produced by the amplification of genomic DNA. cDNA synthesis reactions were done for three biological replicates for the gene expression analysis. An example of agarose gel electrophoresis of the samples analysed by RT-PCR is shown in fig. 9.

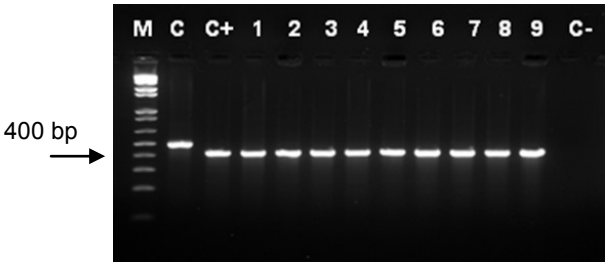

Fig. 9. Control of the cDNA synthesis by RT-PCR of the tobacco lines with the NT-EF primers. **M:** 1Kb plus DNA Ladder (Invitrogen), **C:** genomic DNA control, **C+:** cDNA of non-transformed plant as positive control, **1-3:** non-transformed plants, **4-6:** MZ119 line (prosystemin cDNA). **7-9:** PRO8-56 line (deleted prosystemin). **C-:** water negative control.

The expression of the transgenes was checked by RT-PCR using the Sysdel primers, which amplify both transgenes, the full-length and the deleted prosystemin transcripts, amplifying a fragment of 208 bp. An example of agarose gel electrophoresis is shown in fig. 10.

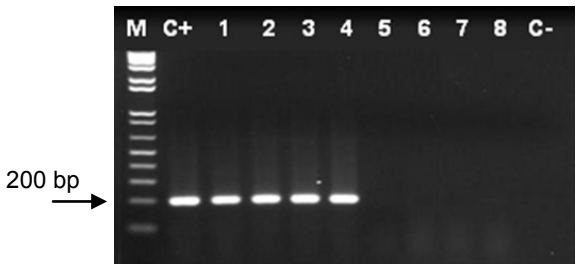

Fig. 10. Agarose gel electrophoresis of RT-PCR of the transgenic lines with Sysdel primers for the control of the expression of the transgenes. **M:** 1Kb plus DNA Ladder (Invitrogen), **C+:** cDNA of MZ transgenic tomato line as positive control, **1-3:** MZ119 line (prosystemin cDNA), **4:** PRO8-56 line (deleted prosystemin), **5-7:** non-transformed plants, **C-:** water negative control

Afterwards, the Prosys primers were used to distinguish the expression of the prosystemin cDNA by RT-PCR, producing a fragment of 110 bp (fig. 11).

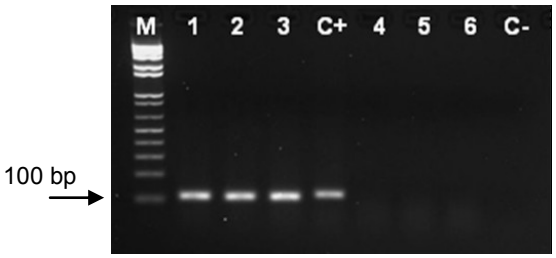

Fig. 11. Agarose gel electrophoresis of RT-PCR of the transgenic lines with the Prosys primers for the control of the expression of the prosystemin cDNA. **M:** 1Kb plus DNA Ladder (Invitrogen), **1-3:** MZ119 line (prosystemin cDNA), **C+:** cDNA of MZ transgenic tomato line as positive control, **4-5:** PRO8-56 line (deleted prosystemin), **6:** non-transformed plants, **C-:** water negative control.

The expression of both prosystemins in tobacco did not affect plant phenotype. The two transgenic lines MZ119 and PRO8-56 did not show any obvious difference to the non-transformed plants NN (fig. 12).

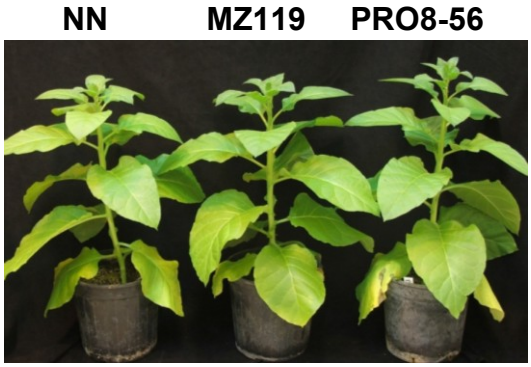

Fig. 12. An example of the phenotype of tobacco transgenic plants, 7-week-old: **NN:** non-transformed plants, **MZ119:** transgenic line expressing the prosystemin cDNA, **PRO8-56:** transgenic line expressing the deleted prosystemin.

5.1.2. Prosystemin positively affected the expression of some defence related genes in tobacco

Three different plants were screened for every transgenic line and three repetitions for every amplification event. The primers used for the Gene Expression analysis by Real Time PCR and some important parameters are listed in table 5. The output data was analyzed, using the Comparative Ct method with the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The housekeeping gene Elongation Factor 1-alpha (EF1-α) a gene expressed at a consistent level in all samples and in every treatment, (Rotenberg *et al*., 2006), it was used as endogenous control to normalize the expression level of the target genes. Non-transformed plants (NN) were set up as calibrator, and therefore, they have an arbitrary RQ value of 1. The RQ of the target genes are then calculated in comparison to the calibrator, to determine if they are down or up-regulated. PCR products were checked on a 2% agarose gel (data not shown), all the products size corresponded to the expected size. Melting curves showed a single amplified product for all genes (data not shown) and the Tm coincided with the Tm calculated in table 3.
Three sets of genes were evaluated. The first set included genes identified by proteomic analysis (Rocco *et al.*, 2008), They are the *RNA-binding protein* (*RBP*), *heat-shock protein* (*HSP*), *stromal ascorbate peroxidase* (*SAP*) and *glutathione S-transferase* (*GST*). All of them are related to plant stress response. The second set included defence-related genes, *calmodulin* (*CaM*), *lipoxygenase* (*Lox1*) and *proteinase inhibitor II* (*Pin II*), all of them linked to the octadecanoid pathway. More precisely, *CaM* and *Lox1* are at the beginning of the pathway and *Pin II* at the end of the cascade of events. Finally, the expression of the two endogenous tobacco prosystemin known as hydroxyproline-rich glycopeptides (*TobHypSys I* and *II*) were also analyzed to verify a possible cross-species activation.
The results indicated that the gene *GST* involved in oxidative stress and in detoxifying many compounds, and *HSP* involved in response to environmental stress, are up-regulated in the transformants, this result is in accordance according to the proteomic study of Rocco *et al.,* (2008). Instead, *RBP* and SAP did not show difference in expression level between transgenic and control plants. Furthermore,

the gene expression of MZ and PRO8 are similar for three genes, but for the *GST* gene. The difference between the MZ and PRO8 lines suggests that the N-terminal region of the prosystemin precursor may contribute to activity detected in tobacco (fig. 13).

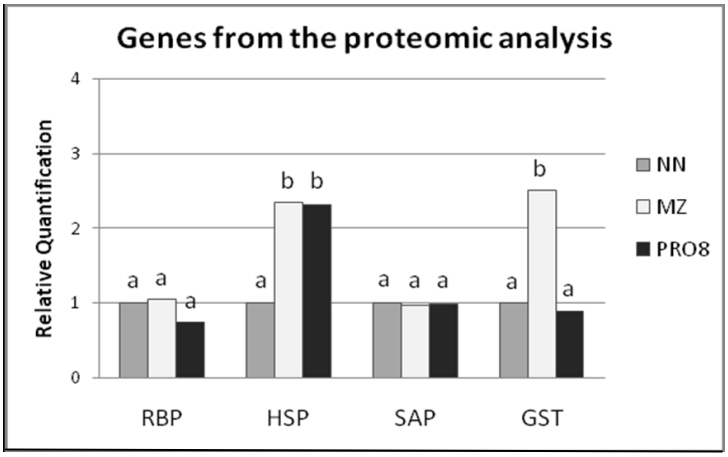

Fig. 13. Relative gene expression of the genes selected from a proteomic analysis. On the *x-axis* are indicated the target genes, **RBP:** RNA-binding protein. **HSP:** low molecular weight heat-shock protein. **SAP:** stromal ascorbate peroxidase. **GST:** glutathione S-transferase. On the *y-axis* is reported the relative quantification. **NN:** non- transformed *N. tabacum* cv. Samsun NN, **MZ:** MZ119 line carrying the prosystemin cDNA. **PRO8:** PRO8-56 line carrying the deleted prosystemin. Different letters indicate significant differences $p < 0.05$.

The second set of genes analyzed is involved in plant response to insects attack. *CaM*, a calcium modulated protein gene, did not show differences in expression compared to the control nor *Lox1*. The protease inhibitor gene *Pin II*, a 'late gene' of the plant response to insects, a protein that inhibits the function of insect peptidases, it was 3-fold up-regulated in the transgenic plants MZ and PRO8. There was not difference between the two transgenic lines, which showed similar gene expression in *CaM, Lox1* and *Pin II*. (Fig. 14).

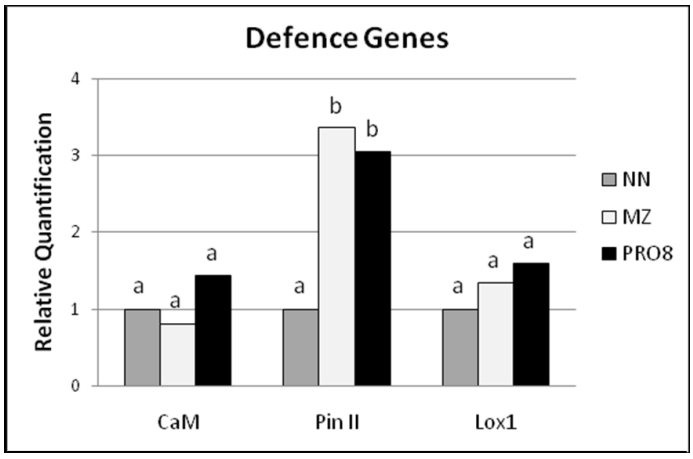

Fig. 14. Relative gene expression of genes related to plant defence. On the *x-axis* are indicated the target genes, **CaM:** calmodulin NtCaM1. **Pin II:** proteinase inhibitor II. **Lox1:** lipoxygenase 1. On the *y-axis* is reported the relative quantification. **NN:** non-transformed *N. tabacum* cv. Samsun NN, **MZ:** MZ119 line with the prosystemin cDNA. **PRO8:** PRO8-56 line with the deleted prosystemin. Different letters indicate significant differences $p < 0.05$.

The relative quantification of tobacco endogenous prosystemins, the hydroxyproline-rich glycopeptides *TobHypSys I* and *II* showed an over-expression in the transgenic lines with mean values statistically different to the control non-transformed plants (fig. 15). Due to the similarity of the coding sequences, primers were designed to amplify the transcripts of both genes. These polypeptides has been reported to have an important role in plant defence and wound response (Ren *et al*., 2008). Overall, the data indicated that the constitutive accumulation of heterologous precursors of a signalling molecule, such as the tomato prosystemin and its mutated version, is related to unpredicted variation in gene expression.

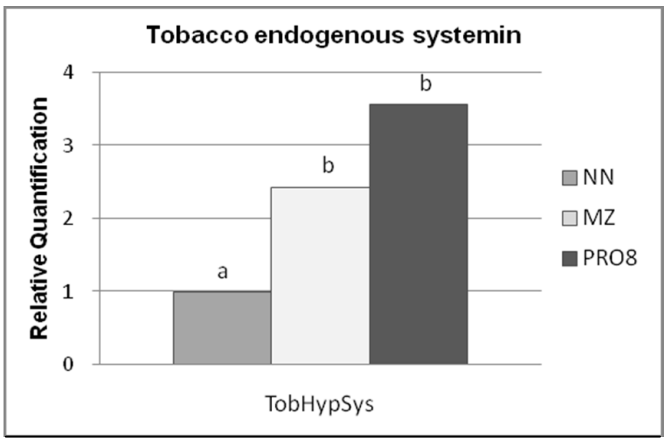

Fig. 15. Relative gene expression of the tobacco endogenous prosystemin-like. On the *y-axis* is reported the relative quantification. **NN:** non-transformed *N. tabacum* cv. Samsun NN, **MZ:** MZ119 line with the prosystemin cDNA. **PRO8:** PRO8-56 line with the deleted prosystemin. Different letters indicate significant differences $p < 0.05$.

Even more, the *TobHypSys* expression of the transgenics MZ and PRO8 are similar, showing both an over-expression with no significant differences at 0.05 level, suggesting that the prosystemin cDNA and the deleted prosystemin provoke a similar effect when expressed in tobacco.

To evaluate a possible biological relevance of the effects of the tomato prosystemin expression in tobacco, the expression level of *HSP, GST, Pin II* and *TobHypSys* was quantified in wounded leaves of the untransformed control plants. Table 6 summarises the results of the expression analysis.

Table 6. Relative quantity (RQ) of the gene expression analysis

Gene	NN	MZ	PRO8
CaM	1.00	0.80	1.44
Pin II	1.00	3.36 *	3.05 *
Lox1	1.00	1.34	1.59
RBP	1.00	1.06	0.75
HSP	1.00	2.36 *	2.32 *
SAP	1.00	0.98	0.99
GST	1.00	2.52 *	0.89
TobHypSys	1.00	2.43 *	3.56 *

* Mean values statistically different to the control NN with $p < 0.05$

The experiment was carried out with 4 week-old plants. Leaves at time zero were harvested, frozen in nitrogen liquid and conserved at -80 until processing. Then, the rest of the leaves were mechanically wounded with a needle as shown in fig.16, and harvested after 6 hours.

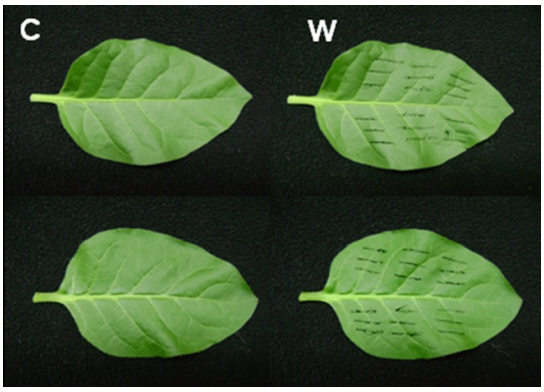

Fig. 16. Leaves of 4-week old tobacco plants: **W:** wounded leaves after 6 hours. **C:** leaves used as control.

The relative quantification (Fig. 17), indicated that the genes *HSP, GST* and *Pin II* are over expressed after wounding. This result is in accordance to the literature (McGurl *et al*., 1994; Rocco *et al*., 2008). The expression of the endogenous tobacco prosystemin-like was not different after wounding. This is explained by the previous evidence that indicated that in wounded leaves, the TobHypSys gene increases after 6 hours until reaching a maximum at 24 hours (Ren *et al*., 2008).

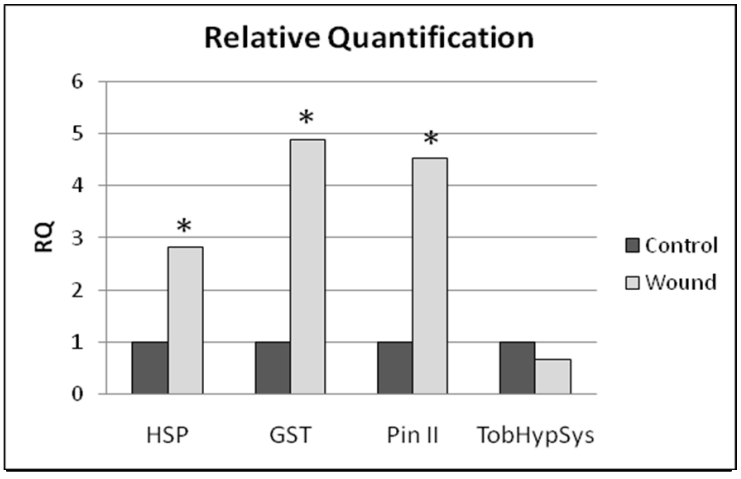

Fig. 17. Relative gene expression after wounding in untransformed plants. On the *x-axis* are indicated the genes, Heat-Shock Protein (**HSP**), Glutathione S-transferase (**GST**), Proteinase inhibitor II (**Pin II**) and **TobHypSys** genes, On the *y-axis* is reported the relative quantification. **Control:** *N. tabacum* cv. Samsun NN, **Wound:** *N. tabacum* cv. Samsun NN after 6 hours wounding. Asterisks indicate significant differences with $p < 0.05$.

The real-time approach, allowed to quantify the effect of wounding after 6 hours. The RQ values of the over-expressed genes of the experiment with the transgenics compared with the RQ values of the last experiment with wounded NN control plants (table 7), showed similar gene level expression for *HSP* and *Pin II*. The data imply

that the tomato prosystemin could provoke an alteration of gene expression level of some genes similar the effect of wounding. However, it is also evident that the effect of wounding (6 hours after) is different from the one due to the transgenes.

Table 7. A comparison of the gene expression of the control plants after wounding and up-regulated genes in unwounded transgenic plants.

Gene	NN		MZ	PRO8
	unwounded	wounded	unwounded	unwounded
HSP	1.00	2.82	2.36	2.32
GST	1.00	4.89	2.81	0.99
Pin II	1.00	4.52	3.36	3.05
TobHypSys	1.00	0.67	2.43	3.56

HSP: heat shock protein, GST: glutathione S-transferase, Pin II, proteinase inhibitor II, TobHypSys: hydroxyproline-rich glycopeptides I and II

Overall, the data indicated that the expression of the tomato prosystemin in tobacco increases the transcription of stress-related genes. Such effect is not only present in the transgenic plants expressing the precursor that includes the systemin peptide, as it was recorded for both types of transgenic lines, MZ and PRO8. However, a difference could be seen in the activation of the GST gene, suggesting that the N-terminal region of the tomato prosystemin also contributes to the activation of gene expression in tobacco. Furthermore, the expression of the native and mutated prosystemin precursors affects the endogenous tobacco HypSys genes. It is difficult to speculate whether such activation is a direct effect of the tomato prosystemin expression or an adaptive response of tobacco to the effects of activation of stress responsive genes in the transgenic plants. Although a systemin-receptor has not been yet identified in tomato, it is not possible to exclude that the prosystemin precursor may undergo multiple processing events in tobacco, which may lead to the direct or indirect activation of oxidative stress mechanisms.

5.1.3. Enhanced resistance of transgenic PRO8 lines against *Botrytis cinerea*

As the gene expression analysis showed that genes involved in plant response to pathogens were over-expressed in the transgenic lines such as *GST* and *HSP*, a fungal resistance bioassay was performed with *Botrytis cinerea*. Five-week old plants were used for the bioassay and four plants for every transgenic line were analysed, a volume of 10 µl of spore suspension (10^6 spores/ml) were inoculated into the leaves in four different points. Lesions were measured at 4, 6 and 8 days after inoculation. An example of attacked leaves at 8 days after inoculation with *B. cinerea* are shown in fig. 18.

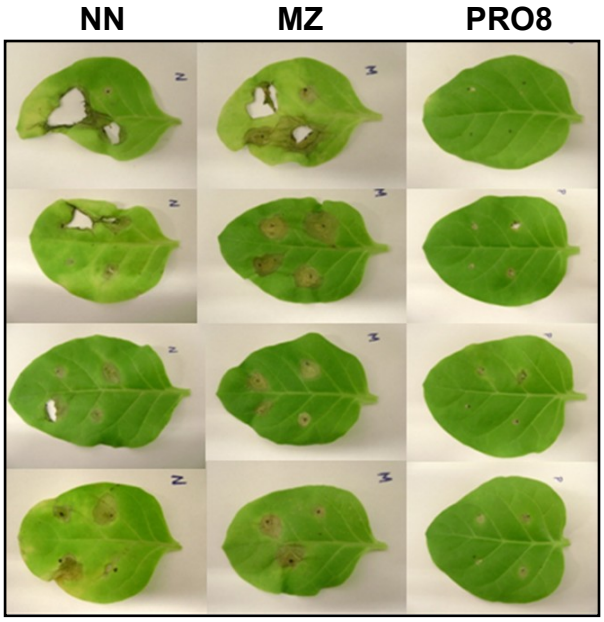

Fig. 18. Leaves of tobacco at 8 days after inoculation with *B. cinerea* (10^6 spores/ml). **NN:** non-transformed control plants, **MZ:** MZ119 line with the prosystemin cDNA. **PRO8:** PRO8-56 line with the deleted prosystemin.

The lesion area provoked by *B. cinerea* in transformed plants (MZ and PRO8 lines) was smaller than the lesions in the non-transformed plants at 6 and 8 days. After analysing the data by one-way ANOVA it was observed that the lesion area after 4 days inoculation, only PRO8 plants were different to the control plants with a significance of 0.05. At 6 days after inoculation, similar results were observed. At 8 days after inoculation, the lesion area of PRO8 is not different to the area of MZ plants. Moreover, PRO8 area is different to control plants (NN). Instead, the lesion area of MZ plants is not different to control plants at the 0.05 level even if the mean lesion area is smaller. Results are shown in fig. 19.

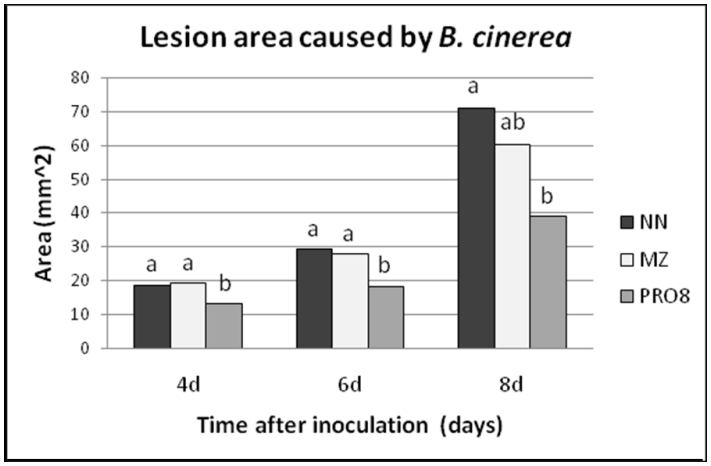

Fig. 19. Lesions area at 4, 6 and 8 days after inoculation. On the *x-axis* is indicated the time in hours after *B. cinerea* inoculation. On the *y-axis* is reported the lesions area in mm^2. **NN:** non-transformed plants, **MZ:** MZ119 line with the prosystemin cDNA. **PRO8:** PRO8-56 line with the deleted prosystemin. Different letters indicate significant differences $p < 0.05$.

The data show an increased resistance against *Botrytis cinerea* in PRO8 transgenic plants. MZ plants seemed to impede in some way the development of the fungus as observed at 8 days after inoculation. These data indicate that the PRO8 transgenic lines have a moderate increase of resistance to the pathogenic fungus *B. cinerea* compare to the MZ lines and the non-transformed plants.

5.2. Expression of the tomato prosystemin cDNA in potato

5.2.1. Plant transformation and molecular characterization of the transgenics

The binary vectors pMZ, containing an expression cassette including tomato prosystemin cDNA under the control of the 35S cauliflower mosaic virus promoter, and pPRO8, a similar binary vector with the prosystemin cDNA missing the systemin encoding exon (fig. 6) were already available at the Prof. Rao Plant Molecular Biology laboratory. These plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 strain. Putative transformed *Agrobacterium* colonies that were able to grew in selective medium, carrying either pMZ or pPRO8, were checked by PCR (not shown) and with the keto-lactose test known as Benedict's test (Fig. 20) based on a metabolic difference between the *Agrobacterium* genus and other bacteria such as *Escherichia spp*.

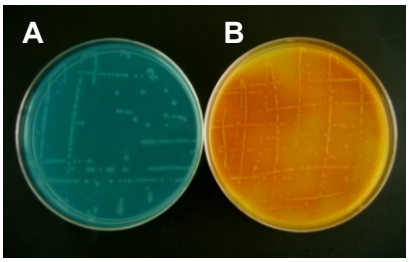

Fig. 20. Keto-lactose test. **A:** *Escherichia coli* cells with the binary vector pPRO8 as negative control . **B:** *Agrobacterium tumefaciens* cells carrying the binary vector pPRO8

Next after, the bacteria carrying the binary vectors pMZ or pPRO8 were used to transform *Solanum tuberosum*, cv. Desiree. An example of the internodes 7 weeks after co-culture is shown in Fig. 21. After calli formation, well-differentiated shoots developeded, were cut and transferred to a selective medium until rooting. Transformants were named after the constructs MZ and PRO8, respectively.

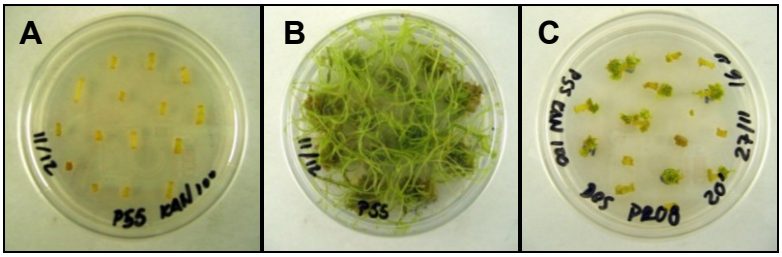

Fig. 21. Potato genetic transformation. *Solanum tuberosum* cv. Desiree with *Agrobacterium tumefaciens* LBA4404 carrying the binary vector pPRO8, 7 weeks after co-culture. **A:** negative control plate, explants without inoculation in selective plates. **B:** positive control plate, explants without inoculation in non-selective plates. **C:** explants with inoculation in selective plates (100 mg/l kanamycin medium)

Putative transformants that were capable to develop roots in selective medium were screened by PCR to detect the presence of the tomato prosystemin transgene. The primers used for the screening were Sysdel primers for the amplification of the prosystemin cDNA and Prosys primers to distinguish the full-lenght prosystemin cDNA. Agarose gel electrophoresis of the PCR positive transformants are shown in fig. 22 and 23.

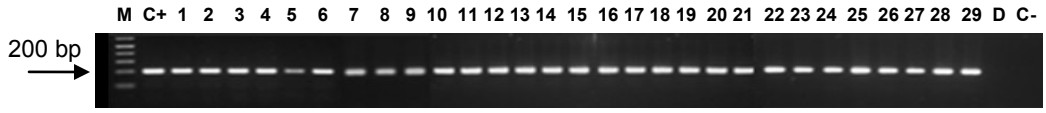

Fig. 22. Agarose gel electrophoresis of PCR of the potato transgenic plants with the Sysdel primers for the control of deleted prosystemin. **M:** 1Kb plus DNA Ladder (Invitrogen), **C+:** PRO8-56 tobacco transgenic line as positive control, **1-29:** PRO8 lines (deleted prosystemin), **D:** non-transformed control, **C-:** water negative control

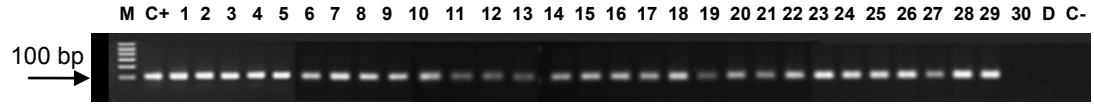

Fig. 23. Agarose gel electrophoresis of PCR of the potato transgenic plants with the Prosys primers for the control of the prosystemin cDNA. **M:** 1Kb plus DNA Ladder (Invitrogen), **C+:** MZ119 tobacco transgenic line as positive control, **1-29:** MZ lines (prosystemin cDNA), **30:** PRO8 line (deleted prosystemin), **D:** non-transformed Desiree as control, **C-:** water negative control

A total of 29 MZ lines and 29 PRO8 lines were positive by PCR analyses. Subsequently, the transcription of the transgenes were controlled by RT-PCR. Positive PCR transformants were transferred *in vivo* in a growth chamber in controlled conditions. After 4 weeks, total RNA was extracted. The synthesis of cDNA was controlled by using the primers Elongation Factor-1 alpha for *S. tuberosum*. The primer pair was designed to anneal on two consecutive exons to detect possible contaminant genomic DNA (not shown). The transcription of both transgenes was controlled using the Prosys and Sysdel primers by RT-PCR. Agarose gel electrophoresis of RT-PCR of the transformants are shown in fig. 24 and fig. 25.

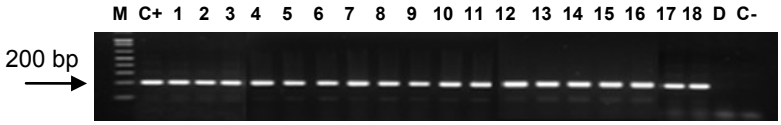

Fig. 24. Agarose gel electrophoresis of RT-PCR of the potato transgenic plants with the Sysdel primers for the control of the deleted prosystemin. **M:** 1Kb plus DNA Ladder (Invitrogen), **C+:** PRO8 line as positive control, **1-18:** PRO8 lines (deleted prosystemin), **D:** non-transformed Desiree as control, **C-:** water negative control

In 13 MZ lines was found that the prosystemin transgene was transcripted and 18 PRO8 lines were positive to the same analysis. All the positive RT-PCR transformants were grown until tuber production. Tubers were collected for further analysis.

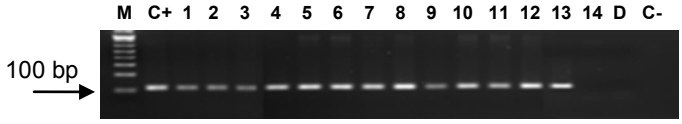

Fig. 25. Agarose gel electrophoresis of RT-PCR of the potato transgenic plants with the PROSYS primers for the control of the prosystemin cDNA. **M:** 1Kb plus DNA Ladder (Invitrogen), **C+:** MZ line as positive control, **1-13:** MZ lines (prosystemin cDNA), **14:** PRO8 line (deleted prosystemin), **D:** Desiree non-transformed control, **C-:** water negative control

The outcome of the potato genetic transformation, including the molecular screening, is summarised in table 8.

Table 8: A summary of the MZ and PRO8 potato transformation and analysis. The percentage refers to the number of internodes used for plant genetic transformation.

	Genotype			
	MZ		PRO8	
	n	%	n	%
Internodes	216	100	198	100
Calli	158	73	170	85
Shoots	91	42	64	32
Rooted shoots	48	22	40	20
PCR positive	29	13	29	14
RT-PCR positive	13/13	-	18/19	-

To control if the tomato prosystemin protein was produced, a western blot was performed using the antibody (Delano *et al*., 1999) supplied by A. Schaller from the University of Hohenheim. The anti-prosystemin raise to the full-length prosystemin. Examples of western blot are shown in fig 26 and 27.

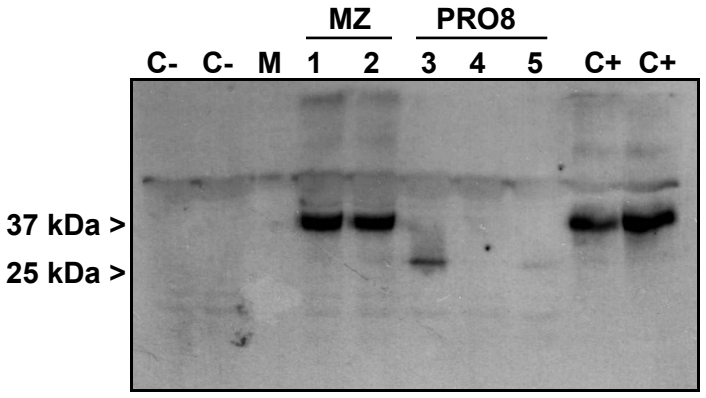

Fig. 26. Western blot analysis of transgenic lines. PRO8 potato lines (deleted prosystemin). MZ potato lines (prosystemin cDNA). **C-:** non-transformed *S. tuberosum* cv. Desiree. **M:** marker Precision Plus Protein (Bio Rad). **1-2:** M4 line, **3:** T13 line, **4:** T47 line, **5:** T48 line, **C+**: tomato plants over-expressing prosystemin cDNA as positive control.

A protein band with an apparent molecular weight of approximately 37 kDa was detected in the tomato positive control (McGurl *et al*., 1994) and in MZ transgenic lines carrying the prosystemin cDNA (fig. 26), which is different from the real molecular weight of 23 kDa. This unexpected migration was previously observed in

mutant prosystemins produced in *E. coli* (Delano *et al*., 1999). The unpredicted migration of prosystemin protein can be explained due to the highly hydrophilic character of the prosystemin peptide that modifies its mobility (Delano *et al*., 1999). A faint band of approximately 25 kDa is also observed in the PRO8 lines. To visualised the truncated prosystemin protein, the experiment was repeated with a higher quantity of total soluble proteins (50 µg), tobacco PRO8 lines were also included (fig. 27).

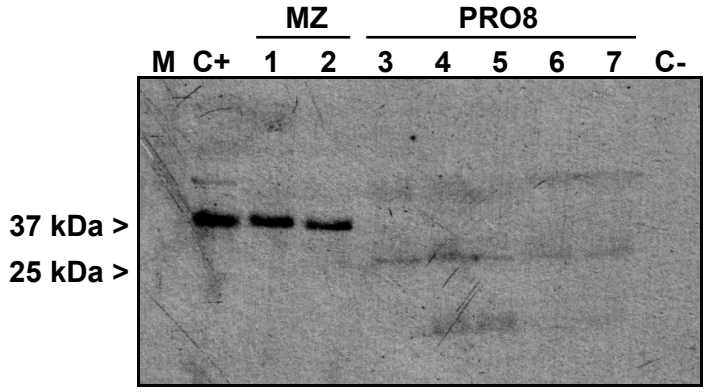

Fig. 27. Western blot analysis of transgenic lines. MZ potato lines (prosystemin cDNA). PRO8 potato lines (deleted prosystemin). **M:** marker Precision Plus Protein (Bio Rad), **C+:** tomato plants over-expressing the prosystemin cDNA as positive control, **1:** M4, **2:** M26, **3:** T13, **4-5:** T16, **6-7:** PRO8-56 tobacco line. **C-:** non transformed *S. tuberosum* cv. Desiree.

The tomato positive control and the MZ lines presented the protein band of about 37 kDa and a protein band of about 25 kDa band was visible again in lanes 3, 4, 5, 6, and 7, all PRO8 potato and tobacco transgenic lines. The band was absent in the non-transformed control plant.

5.2.2. Prosystemin transgenic plants showed a specific effect in defence gene expression

For the gene expression analysis plants were grown from tubers in a growth chamber under controlled conditions, leaves were collected from 4-week old plants. The two transformed genotypes MZ and PRO8 were screened together with non-transformed plants *S. tuberosum* var. Desiree used as a control. Three PRO8 lines and three MZ lines were selected according to the tubers homogeneity. Transgenic plants did not show any visible difference in phenotype compared to the non-transformed plants, nor the tubers (not shown). An example of potato transgenic plants MZ and PRO8 is shown in fig 28.

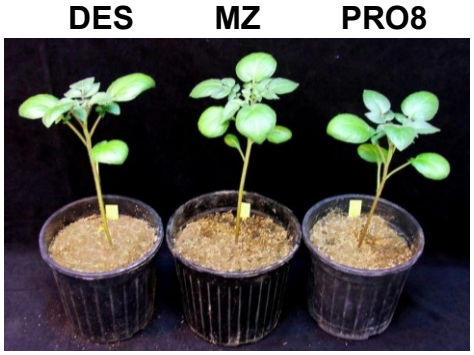

Fig. 28. Potato transgenic plants 4-week-old: **DES:** *S. tuberosum* cv. Desiree, **MZ:** transgenic plant expressing the prosystemin cDNA, **PRO8:** transgenic plant expressing the deleted prosystemin.

Total RNA was isolated from leaves of every plant, three plants for every transgenic line, a total of 27 plants were screened by Real Time PCR and RNA was extracted from all of them. Agarose gel electrophoresis of total RNA of some samples is shown in fig. 29.

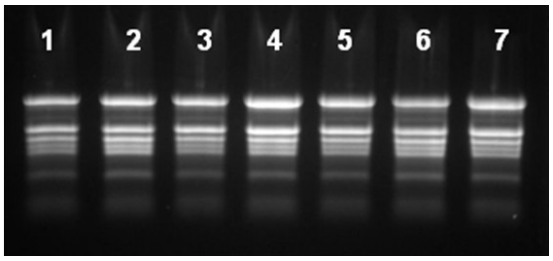

Fig. 29. Agarose gel electrophoresis of total RNA from potato leaves (4µg/lane) **1-4:** MZ lines expressing the prosystemin cDNA, **5-7:** PRO8 lines expressing the deleted prosystemin.

After mRNA retro-transcription, the quality of cDNA was controlled by RT-PCR with the primers Elongation Factor 1-*α* for *S. tuberosum* to control a possible contaminant DNA. An example of gel electrophoresis of RT-PCR with the EF1-α primers is shown in fig. 30.

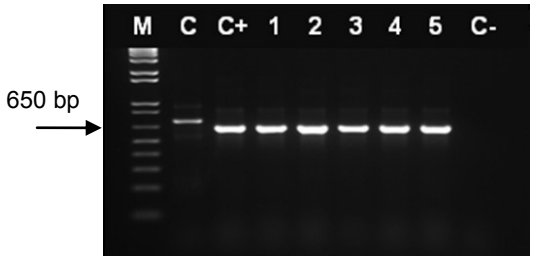

Fig. 30. Control of the cDNA synthesis, RT-PCR of the potato lines with the EF1-*α* primers. **M:** 1Kb plus DNA Ladder (Invitrogen), **C:** genomic DNA of non-transformed plant as control, **C+:** cDNA of non-transformed plant as positive control, **1-5:** PRO8 lines expressing the deleted prosystemin. **C-:** water negative control.

The transcription of the transgenes was controlled again, as before, by RT-PCR with Prosys and Sysdel primers. The Elongation Factor 1-alpha (EF1-α) gene was used as the endogenous reference to normalize the expression level of the genes being analysed, as EF1-α was found to be at a consistent level in all samples (Nicot *et al.*, 2005). Non-transformed plants cv. Desiree were used as calibrator. Ct values were used to calculate the relative quantity (RQ value) of every target gene by using the formula $2^{-\Delta\Delta Ct}$. PCR products were also checked on a 2% agarose gel (not shown). From the dissociation curves analysis, melting curves (not shown), confirmed a single amplified product and the Tm corresponded with the Tm calculated in table 5.
Three set of genes were analysed by Real Time RT-PCR, all the genes are related to plant defence. The first set included *calmodulin* (*CaM*), *hydroperoxide lyase* (*HPL*), and *lipoxygenase 3* (*Lox3*), all 'early genes' associated with the octadecanoid pathway. *CaM* and *Lox* involved to the generation of the 13-hydroperoxy octadecatrienoic acid and subsequent generation of 12-OPDA, and the *HPL* gene essential for the generation of VOCs. The second set of genes included pathogen related genes, the *pathogenesis-related protein1b* (*PR1b*) and the *endo-1,3-beta-D-glucanase* (*GluB2*). The last set consisted in the endogenous potato prosystemins 1 and 2 (*PotProsys*) and the endogenous hydroxyproline-rich glycopeptides (*PotProHypSys*), peptides involved in plant response after wounding.

The expression of the *CaM* and *HPL* genes did not show differences in the transgenics lines respect to the non-transformed control (fig. 31). Instead, the gene *Lox3* was 11-fold over expressed in the MZ lines and 6-fold over expressed in the PRO8 lines respect to the non-transformed control plants (DES).The expression of the transgenic lines did not showed an importance difference between MZ and PRO8.

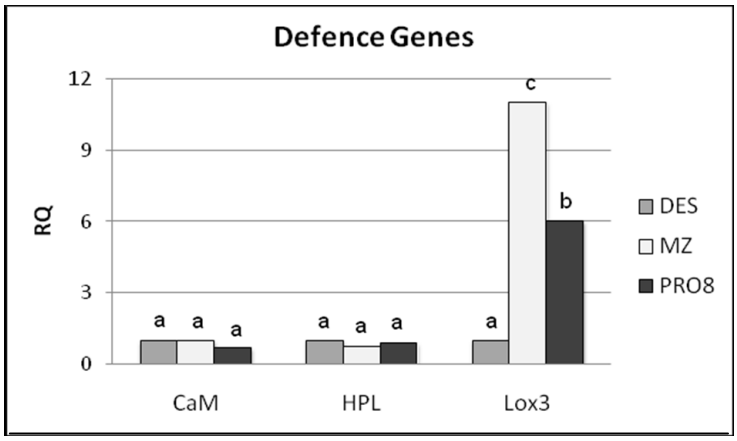

Fig. 31. Gene expression analysis of defence genes. On the *x-axis* are indicated the target genes, **CaM:** calmodulin. **HPL:** fatty acid hydroperoxide lyase. **Lox3:** lipoxigenase 3. On the *y-axis* is reported the relative quantification. **DES:** non transformed *S. tuberosum* cv. Desiree, **MZ:** MZ lines with the prosystemin cDNA. **PRO8:** PRO8 lines with the deleted prosystemin. Different letters indicate significant differences $p < 0.05$.

These results could indicate that the N-terminal prosystemin proteins plays a role in potato in activation of some defence genes. The over-expression of *Lox3*, an 'early gene' in both transgenic lines suggests the induction of some early genes at the beginning of the signal cascade.
Two genes related with pathogen response were also analysed, *GluB2* and *PR1b*. The *PR1b* gene showed some down regulation in the PRO8 lines, and *GluB2* showed a difference in expression in MZ lines compared to the control. Even though, after the statistical analysis the data did not show a significant difference (fig. 32).

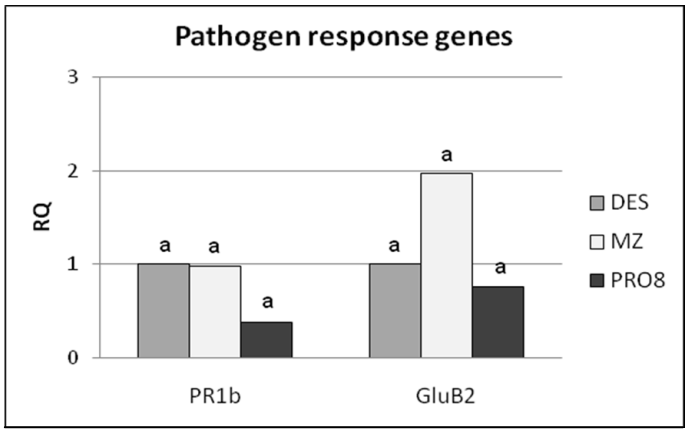

Fig. 32. Gene expression analysis of pathogen response genes. On the *x-axis* are indicated the target genes. **PR1b:** pathogenesis-related protein 1b precursor. **GluB2:** 1,3-beta-D-glucanase. On the *y-axis* is reported the relative quantification. **DES:** non transformed *S. tuberosum* cv. Desiree, **MZ:** MZ lines with the prosystemin cDNA. **PRO8:** PRO8 lines with the deleted prosystemin. Different letters indicate significant differences $p < 0.05$.

The two endogenous potato prosystemins (*PotProsys 1* and *2*) were analysed together due to the high similarity of the two sequences. The potato prosystemins and the hydroxyproline-rich glycopeptides of potato (prosystemins-like peptides) did not show a difference in the expression level of both transgenics compared to the non-transformed control, suggesting that the tomato prosystemin do not produce an activation of the endogenous potato prosystemins genes involved in plant defence (fig. 33).

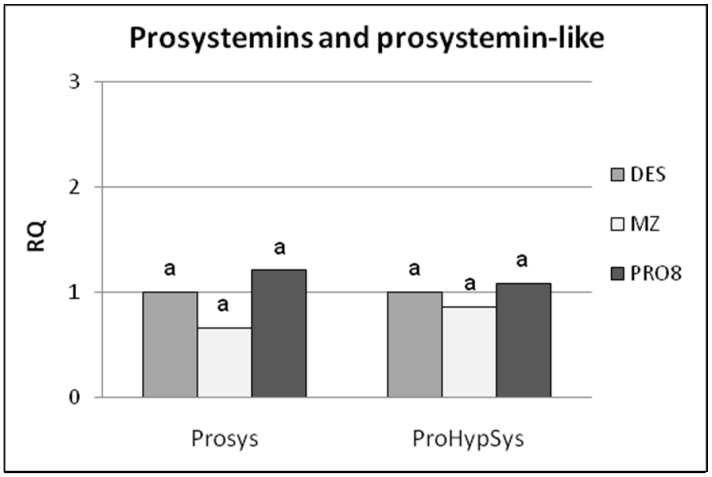

Fig. 33. Gene expression analysis of the potato endogenous prosystemins and the potato hydroxyproline-rich glycopeptide. On the *x-axis* are indicated the target genes, **Prosys:** potato endogenous prosystemin 1 and 2. **ProHypSys:** hydroxyproline-rich glycopeptide. On the *y-axis* is reported the relative quantification, **DES:** non-transformed *S. tuberosum* cv. Desiree, **MZ:** MZ lines with the prosystemin cDNA. **PRO8:** PRO8 lines with the deleted prosystemin. Different letters indicate significant differences $p < 0.05$.

Previously, it has been show that the tomato systemin, a potent activator of the octadecanoid pathway produces the systemic activation of defence genes by amplifying the jasmonic acid in tomato. When expressed in potato, systemin seems having a specific effect. All the genes analysed were related to plant defence, but only *Lox3* was up-regulated, as the expression of the other genes did not show significant differences. These results suggest that the tomato prosystemin have, in potato, at least partially, an effect similar to the one recorded in tomato. The results are then consistent to what reported by Narváez-Vásquez and Ryan (2002).

6. DISCUSSION AND CONCLUSIONS

There is a continuing need to increase agricultural production and major losses are due to pests. The presence of pests and/or pathogens also increases the energy input and labour required in agriculture. Furthermore, massive applications of agro-chemicals may also result in adverse effects to the environment. Integrated Pest Management (IPM) is a tool for sustainable agriculture, an approach that includes the development of resistant cultivars and the introduction of environmentally friendly bio-pesticides.

The study of genes and signals that are involved in the modulation of plant defence against pests is an area of research that has both basic and practical implications. A component of IPM is the genetic engineering of crops to develop transgenic plants with different insecticidal genes. For instance, genes that confer resistance to insects have been inserted into different plant species. Genetic modified plants expressing insecticidal proteins (Cry toxins) from *Bacillus thuringiensis* (*Bt*) were first commercialized. *Bt*-transgenic plants have become important components of maize and cotton IPM programs worldwide (Schelton *et al*., 2008). Furthermore, transgenic plants expressing trypsin inhibitor gene have been developed in tobacco and others (De Leo *et al.*, 2001). Moreover, transgenic tobacco, maize and rice expressing lectin genes have shown adverse effects against several insect species feeding on these crops. Also, transgenic tobacco plants expressing chitinase gene have shown increased resistance to a pathogen attacks (Zhu *et al.,* 1994).

At present, new insecticide molecules are being developed for expression in plants and plant genes are being altered to affect biochemical pathways that elicit insect resistance (Shelton *et al.*, 2008). However, less attention has been given to strategies aiming at increasing the natural occurring plant defences against pests and in general biotic stress.

Tomato responds to herbivore attack by releasing a mobile peptide called systemin which is derived from a 200-amino acid precursor called prosystemin. Systemin is released from wound sites of tomato leaves as a consequence of mechanical wounding or insect attacks (McGurl *et al*., 1992). After the discovering of systemin in tomato, homologues were isolated from potato, nightshade and bell pepper (Constabel *et al.* 1998), but not in tobacco, an evolutionary more distant species. In tobacco, a prosystemin hortologue has been isolated encoding for two peptides, hydroxyproline-rich systemin (*TobHypSys*) I and II, they are active in inducing proteinase-inhibitor synthesis (Pearce *et al.*, 2001). These peptides do not share any structural similarity with tomato systemin, although they play similar roles also in the rapid activation of a 48 kDa MAPK and in the alkalinization of tobacco cell culture medium. The 165-amino acid pro-hormone protein is inducible by treating tobacco plants with methyl jasmonate, indicating a mode of action involving the octadecanoid signalling pathway, as in the tomato systemin system (Ryan and Pearce, 2003).

In the present work, the effect of prosystemin gene was studied in two Solanaceae such as tobacco and potato to increase the understanding of about a possible biotechnological function of the prosystemin as enhancer of plant endogenous resistance in different plant species. In order to better characterize the impact of the tomato prosystemin cDNA on the expression of tobacco and potato defence genes, transgenic plants expressing a prosystemin mutated allele missing the systemin encoding exon were also studied. Transgenic plants were characterized and the expression of a set of genes involved in plant defence was analysed. The modification in gene expression registered between transgenic tobacco expressing

the tomato prosystemin gene and the mutated allele is reported and discussed together with the possible function of the N-terminal region of the prosystemin protein precursor in the activation of the defence response.
Real time PCR was used to determine the gene expression analysis in the transgenic plants MZ (expressing the full-length prosystemin cDNA) and PRO8 (expressing the deleted prosystemin) in both species of Solanaceae.
The analysis of the tomato prosystemin in tobacco showed that prosystemin affects the expression of some stress and defence-related genes such as *HSP*, *GST*, *Pin II* and *TobHypSys* in tobacco plants. *GST* is involved in oxidative stress, HSP are a class of functionally related proteins whose expression is increased when cells are exposed to elevated temperatures or many other stress, consequently the HSP are also referred as stress proteins (Scarpeci *et al*., 2008). The gene expression analysis confirmed that the tomato prosystemin up-regulates some stress related genes, this in accordance with the proteomic approach of Rocco and others (2008). The authors found that tobacco plants expressing the prosystemin gene showed a higher number of proteins involved in plant defence against pathogens and oxidative stress. Comparing the proteomic data with the transcriptional analysis, the HSP and GST proteins were over-synthesized in MZ tobacco transformants.
In addition, the present work indicated that the effects in tobacco involve also other classes of genes. Transgenic plants showed an up-regulation of protease inhibitor II (Pin II), which are considered to function as important antiherbivore defences (Pearce *et al.,* 1993). However, two early genes of the octadecanoid pathway *Lox* and *CaM* were not over-expressed in transgenic plants. Hence, the data suggest that the possible accumulation of PI proteins is not due to a classic systemin-dependant response, as it occurs in tomato. In tomato, the constitutive expression of the prosystemin precursor provokes an strong accumulation of PI in leaves (McGurl *et al*., 1994).
The prosystemin also affects the expression of *TobHypSys* in tobacco. The TobHypSys I and II precursor has been found to be induced by mechanical and herbivorous wounding (Rocha-Granados *et al*., 2005) but their response to other stresses has not been yet studied. Ren and Lu (2006) showed that transgenic plants over-expressing the TobHypSys-A precursor improved the resistance against *Helicoverpa armigera* in tobacco by inducing PIs and PPO activity. In the future it will be interesting to further explore the possibility that transgenic tobacco lines have increased level of resistance to herbivorous pests.
The gene expresion analysis indicated that the constitutive accumulation of heterologous precursor of a signalling molecule, such as the prosystemin, is associated with unexpected gene expression changes. It is difficult to speculate whether is the tomato prosystemin that directly activates some defensive pathways in tobacco, or its possible proteolytic products. A previous study demonstrated that a peptide engineered in the tomato prosystemin was released in transgenic tobacco plants constitutively expressing this chimeric precursor (Tortiglione *et al*., 2003). The data suggested that tobacco plants are able to process the tomato prosystemin. However, the possible presence of other products released from the multiple processing of the precursors, or by the recognition of other sites that flank the di-basic sites involved in systemin release, was not investigated.
The processing of the prosystemin is still unknown in tomato. Surprisingly, the constitutive accumulation of the systemin precursor is associated to a constitutive accumulation of defensive products, despite that it has long been proposed that prosystemin processing (i.e. the released of the bioactive systemin peptide) is

wound-inducible (McGurl *et al*., 1992, 1994). Therefore, a possible processing of the tomato prosystemin in tobacco in absence of wounding would not be in contradiction to what occurs in tomato.
Another limitation is that it has not been yet identified a systemin receptor in tomato. Early studies by the Ryan group identified a possible candidate in the SR160 (Scheer and Ryan, 1999). Further studies indicated that the SR160 systemin-binding protein was the tomato brassinosteroid receptor (BRI1) (Scheer and Ryan, 2002). More recently, the role of SR160/BRI1 as systemin receptor has been questioned by independant reserchers. Currently, it is believe that the SR160/BRI1 binds sytemin, but it is not able to initiate signal transduction in tomato (Scheer *et al*., 2003; Holton *et al*., 2007; Malinowski *et al*., 2009). The finding that the expression of the tomato SR160/BRI1 is able to create a systemin-dependent medium alkalinisation in tobacco, has also been questioned (Scheer *et al*., 2003). Interestingly, rapid responses to systemin in tobacco cells are not due to the tomato BRI1 receptor, systemin is able to induce the alkalinisation of the medium of tobacco cell culture (Malinowski *et al*., 2009), the authors also discussed the possible presence of a tomato homologue of the unknown systemin receptors. The present gene expression analysis indicated that similar effects are present in both the tobacco transformants expressing the full-length precursor and the precursor lacking the systemin fraction. Therefore, it is unlikely that the activation of gene expression are mainly due to the possible released of the systemin peptide. Moreover, the experiments with non-transformed wounded plants showed that the modification in gene expression of some genes (*HSP* and *Pin II*) is similar to the effect provoked by wounding.
The fungal resistance bioassay with *Botrytis cinerea* showed that PRO8 transformants carrying the deleted prosystemin have a moderate increase in resistance in comparison to the control plants, which is consistent with the present gene expression analysis and with the proteomic study of tobacco transformants expressing the deleted prosystemin (Rocco M. unpublished) that showed an increasing of proteins in transformant plants compared to non-transformed control plants. MZ plants carrying the full-length prosystemin cDNA showed smaller area lesions provoked by the fungus *B. cinerea*, even if a statistical difference was not observed after 8 days inoculation. Although, the presence of significant damaged leaf area did not allow an accurate measurement at more time points, the bioassay indicated that differences between MZ plants and control plants seemed to increase with the time.
The expression of the tomato prosystemin gene in tobacco enhances the transcription of stress-related genes. The effect is detected in the transgenic plants expressing the full-length precursor including the systemin peptide and the deleted prosystemin lacking the systemin coding exon. Nevertheless, the GST gene was up-regulated only in the transformants carrying the full-length precursor. The data propose that the modification in gene expression is not only due to the *Sys* sequence and that the N-terminal region of the tomato prosystemin also contribute to the activation of gene expression in tobacco. Dombrowski and others (1999) showed that the absence of the systemin sequence in the tomato prosystemin gene eliminate the induction of proteinase inhibitor in tomato. Here we report that the C-terminal of the precursor is also active, implying a significant difference between the function of the prosystemin precursor in tomato and tobacco.
The gene expression analysis of transgenic potato plants showed that the tomato prosystemin gene in potato does not affect pathogen-related genes as *endo-1,3-beta-D-glucanase* (*GluB2*) and *pathogenesis-related protein1b* (*PR1b*), nor the

defence-related potato endogenous systemins, *Prosys* and *hydroxyproline-rich glycopeptides* (*ProHypSys*) genes, Also, the defence-related genes such as *calmodulin* (*CaM*) and *hydroperoxide lyase* (*HPL*) did not show any modification in expression. Only *Lox3* was over-expressed in both transformant plants. *Lox*, an early gene of the octadecanoid pathway, is involved in the production of the 13-hydroperoxy octadecatrienoic acid and subsequent production of the 12-OPDA, a essential compound for the generation of jasmonic acid (Walling, 2000). Antisense expression of Lox3 in *Nicotiana attenuata* showed a decreasing of defence response such as the accumulation of jasmonic acid and the consequent production of trypsin protease inhibitors (Halitschke and Baldwin 2003), indicating that Lox3 supplies fatty acid hydroperoxides as substrates for jasmonates synthesis, a key signal for the transcription of defensive proteins, an effect that is coherent to what was reported by Narváez-Vásquez and Ryan (2002). The authors found that the expression of the prosystemin cDNA in potato regulated the synthesis of PIs in leaves and major storage proteins in tubers. The results are consistent with Orozco-Cárdenas and others (2001) and Ryan and Pearce (2003) that showed that the systemin, provoke the systemic activation of defence genes by amplifying the jasmonic acid. When prosystemin is expressed in potato, it has a partially similar effect. The two transformants carrying either the full-length prosystemin or the mutated prosystemin showed an over expression in *Lox3*, indicating that the N-terminal prosystemin proteins plays a role in potato in the activation of *Lox3*.

Our work along with previous evidence present in the literature indicates that the systemin signalling pathway of tomato is a unique example among Solanaceae plants, with the known exception of potato (Narváez-Vásquez and Ryan, 2002).

In conclusion, the results of this study demonstrate that tobacco plants expressing tomato prosystemin have a modified expression of genes involved in plant defence response, which is also confirmed by the moderate tolerance shown by transgenic plants to the pathogenic fungus *B. cinerea*. Viceversa, in potato the constitutive expression of tomato prosystemin is associated with the up-regulation of only *Lox3* gene, suggesting a specific effect of prosystemin in potato. Overall, the data support the biotechnological role of tomato prosystemin as enhancer of the plant endogenous defence against biotic stresses.

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Poster Abstract – C.18

STUDY OF THE EFFECT OF TOMATO *PROSYSTEMIN* GENE EXPRESSION IN TOBACCO PLANTS

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Nicotiana tabacum, transgenic plants, defence genes

Systemin, an octadeca-peptide isolated from tomato is the signalling molecule involved in the local and systemic wound response. It derives from the C-terminal region of prosystemin, a prohormone of 200 amino acids (McGurl and Ryan, 1992). Prosystemin orthologues have been found in other *Solanaceae* species such as potato, bell pepper and black nightshade, all members of the *Solaneae* subtribe, whereas they were not detected in tobacco, which belongs to the *Nicotianae* subtribe. Tobacco systemins, two hydroxyproline rich peptides released from the same precursor, are functionally related to tomato systemin but do not share with it sequence similarity. Morover Prosystemin gene does not share any identity with the tobacco and tomato HP-rich systemin precursors. Although tobacco does not respond to externally applied tomato systemin (Scheer et al, 2003), it has been shown recently that the constitutive expression of the tomato prosystemin gene in tobacco is associated with a significant increase of the amount of a number of proteins involved in the protection of plants from pathogens and oxidative stress (Rocco et al., 2008). In order to better characterize the impact of the tomato prosystemin gene on the expression of tobacco defence genes, a new set of transgenic plants expressing a systemin mutated allele missing the systemin encoding exon, were produced. Transgenic plants were fully characterized and analyzed as far the expression of a set of genes involved in plant defence. The modification in gene expression registered between transgenic tobacco expressing the tomato prosystemin gene and the mutated allele is reported and discussed along with the possible role of the N-terminal region of the prosystemin protein precursor.

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STUDIO DELL' EFETTO DELL' ESPRESSIONE DELLA PROSISTEMINA DI POMODORO IN PIANTE DI TABACCO

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Nicotiana tabacum, piante transgeniche, geni di difesa

La sistemina, un octadeca-peptide isolato da pomodoro, è una molecola segnale che fa parte del sistema locale e sistemico di risposta alla ferita e danno meccanico. Esso deriva dal procesamento della regione C-terminale della prosistemina, un pro-ormone di 200 residui (1), ma il ruolo della regione N-terminale del precursore non e' ancora definito. Ortologhi della prosistemina sono stati trovati in altre piante della famiglia *Solanaceae* per esempio patata, peperone ed erba morella, tutti della *subtribae Solaneae*; tuttavia la sistemina non è stata individuata nel tabacco, che appartiene alla *subtribae Nicotianae*. Le sistemine del tabacco, due peptidi ricchi di idrossiprolina, rilasciati da un unico precursore proteico, sono funzionalmente simili alla sistemina del pomodoro, ma strutturalmente diversi. Sebbene il tabacco non risponda al trattamento esterno con la sistemina di pomodoro (2), di recente è stato dimostrato che l'espressione costitutiva del gene della prosistemina di pomodoro è associata ad un incremento delle proteine coinvolte nella difesa della pianta contro patogeni ed anche nello stress ossidativo (3). Per caratterizzare meglio l'impatto del gene della prosistemina di pomodoro nella espressione di geni di difesa in tabacco, sono state prodotte delle piante transgeniche che esprimono l'allele mutato della prosistemina privo dell'esone codificante per la sistemina. Le piante transgeniche sono state completamente caratterizzate ed analizzate per quanto riguarda l'espressione di un gruppo di geni coinvolti nella difesa della pianta. Le differenze di espressione genica tra le piante transgeniche che esprimono il gene della prosistemina del pomodoro e l'allele mutato sono quindi da ascrivere ad un possibile ruolo della regione N-terminale del precursore.

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4-7

A proteomic approach to study peptide signalling in plant defence: convergence and differences in *Solanaceae* species.

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Recently, different studies have demonstrated that peptide signalling plays an important role in various aspects of plant growth, development and defence responses. Among the known signals known, the 18-amino acid systemin peptide is one of the best characterised. Systemin is a defence signal in *Solanaceae* family members, activating genes involved in direct and indirect resistance against herbivorous pests. In tomato, systemin is released from proteolytic cleavage of a 200 amino acid precursor (prosystemin, PS) that lacks signal peptides and is though to be cytosolic. The biological activity of PS resides exclusively in the systemin domain, although it is not excluded that the repeated sequences characterising the PS primary sequence may be involved in signalling processes. Tobacco plants that, like tomato, belong to the Solanaceae family, exhibit a systemic wound response similar to that of tomato, but a PS ortholog has not been found. Tobacco possesses two hydroxyproline-rich glycopeptide systemin (TobHS) precursor proteins known as preproTob- HypSys-A and B. Very little is know about prohormone processing in plants and specifically, whether or not there are conserved mechanisms across species. For instance, it has been proposed that the tomato PS may be correctly processed in tobacco. To unravel a possible convergence in peptide signalling across species of the Solanaceae family, we expressed the tomato PS and a recombinant truncated form lacking the systemin sequence in tobacco. By using a combination of proteomic and gene expression analyses, we demonstrate that the constitutive expression of the tomato prosystemin gene significantly affected host protein synthesis. Variation in the proteome was also present in plants that express the truncated PS, suggesting that, in accordance to the literature, the effects are not due to systemin sequence. This is also consistent to the fact that the overlap between the repertoire of genes activated by prosystemin overexpression in tobacco and in tomato is very limited. Many of the identified differentially expressed genes are related to energy metabolism, oxidative stress as well as pathogen/virus response. The transcriptional analysis by real-time PCR indicated that differences in gene expression are limited. These variations in gene expression can account for the moderate increased resistance of the transgenic lines to the pathogenic fungus *Botrytis cinerea*. This study indicates that the constitutive accumulation in tobacco of heterologous precursors of a signalling peptide is associated with unexpected differences in gene expression, implying that some processing events may be conserved but that PS signalling is species specific.

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Molecular characterisation of Vesuvian apricot cultivars: implications for the certification and authentication of protected plant material

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SUMMARY
In the EU, the production of local varieties with well-known quality characteristics may be defended by a Protected Designation of Origin (PDO) or a Protected Geographical Indication (PGI) label. Although the award of either status is based on meeting a number of conditions, the registration, distinction, and protection of such plant material is based on morphological traits. Here, using seven SSR and four AFLP primer pairs, we report on the molecular characterisation, of a group of 36 traditional apricot (*Prunus armeniaca*) varieties from southern Italy. This group included all the varieties approved for the "Albicocca Vesuviana" (Vesuvian apricot) PGI label. Cluster analysis, based on genetic distances, clearly differentiated the 11 PGI cultivars from the other genotypes. Nonetheless, among the 11 PGI-cultivars, two pairs were found to have identical SSR and AFLP profiles. In addition, the molecular analysis indicated the presence of mis-labelling and erroneous denominations of trees from the PGI area. The data revealed that DNA fingerprinting should be always deployed to complement the use of morphological traits in the description of plant material during the institution of a PDO or PGI label, and to evaluate the presence of genetic inconsistencies in orchards.

Apricot, *Prunus armeniaca* L. (2n = 16), is a member of the family *Rosaceae* and was probably domesticated in western China and Central Asia. According to UN FAO estimates (http://www.fao.org/corp/statistics/en/), apricot cultivation is spread throughout the World. All major apricot-producing countries lie around the Mediterranean basin and in the Middle East, with the exception of Japan. Italy is the third largest producer in the World, after Turkey and the Islamic Republic of Iran. Approx. 40% of Italian apricot production comes from the Campania region, where the most important area for apricot cultivation lies around Mount Vesuvius. Despite yearly fluctuations in yield, this area accounts for approx. 60% of all regional production, but ≤ 20% of Italian production (Mainolfi *et al.*, 2006). A considerable amount of apricot fruit (≤ 80%) is processed locally and sold canned, in syrup, or used for nectars, juices, jams, and pastes. Vesuvian apricots have superior characteristics (e.g., high sugar content, uniform colour, proper texture, pulp yield, and a rich flavour) that make them well-suited for processing and as ingredients in traditional confectioneries and pastries (Forlani and Pugliano, 1997). The remaining apricot fruit production is distributed to local markets (Mainolfi *et al.*, 2006), as Vesuvian apricots become marketable later than fruit imported from other countries. Unfortunately, imported apricots are becoming more widespread in this industrial sector because of their more regular availability. In addition, the area devoted to apricot cultivation around Mount Vesuvius is gradually decreasing, mainly because of the urbanisation of rural areas. The vast majority of orchards are small, family-businesses (≤ 5 ha), making it difficult to increase profits through technology-based improvements in productivity.

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The region of cultivation, and cultivar, are important factors that influence the levels of carotenoids in apricot fruit, which are usually higher in cultivars grown in the Mediterranean region (Dragovic-Uzelac *et al.*, 2007). In order to protect Vesuvian apricot production, an EU Protected Geographic Indication (PGI) is shortly to be registered ("Albicocca Vesuviana"). This trade name will be crucial for the promotion of a product with superior characteristics, the maintenance of biodiversity, improvements to the incomes of farmers (in return for a "genuine effort to improve quality"), the preservation of rural areas around Mt. Vesuvius, and to give accurate information about the origin of a product that is easily identifiable by local consumers.

Although several apricot cultivars have been described in the Vesuvian area (Forlani and Pugliano, 1997; Forte, 1987), only 11 will be accepted for the production of PGI-certified fruit. These traditional cultivars result from selections by local peasants, are particularly adapted to the Vesuvian environment (Massai and Pennone, 2007), and probably represent a distinct genetic pool. This would be consistent with the evidence that genetic differentiation of apricot is linked mainly to its geographical origin (De Vicente *et al.*, 1998; Hagen *et al.*, 2002; Hormaza, 2002; Zhebentyayeva *et al.*, 2003).

The present study describes the molecular characterisation of 36 apricot cultivars grown in the Vesuvian area, including the 11 to be granted the "Albicocca Vesuviana" (Vesuvian Apricot) PGI trade-name. As genetic inconsistencies can be specially relevant in traditional plant material, our aims were: i) to study genetic relationships; and ii) to identify allelic combinations that could distinguish the PGI cultivars from other locally-grown cultivars.

MATERIALS AND METHODS

Plant material

We analysed two trees of each of the 11 *P. armeniaca* cultivars to be included in the PGI trade-name, and present at the Istituto Sperimentale per la Frutticoltura, Sezione Operativa Periferica di Caserta. The 11 cultivars were (known synonyms in brackets): 'Barracca' (Baracca), 'Boccuccia Liscia', 'Boccuccia Spinosa', 'Ceccona' (Pelese Eugenio), 'Fracasso' (Bocuccia di Fracasso, Bocuccia 'ntuppecosa), 'Monaco Bello' (Prete Bello), 'Palummella', 'Pellecchiella', 'Portici', 'San Castrese', and 'Vitillo'. In addition, we analysed the most common apricot types grown in the Campania region: 'Ananassa' (Nanassa), 'Biotipo AB05', 'Cafona' (Cafone), 'Carpona', 'Cristiana', 'Diavola', 'Grangicano', 'La Signora', 'Paolona', 'Piciona', 'Pollastrella', 'Portuallara', 'Puscia', 'S. Antonio', 'S. Giorgio', 'Scequariella', 'Schiavona', 'Scialo', 'Sel. 2 Portici', 'Sel. 5 Portici', 'Silvana', 'Sonacampana', 'Stella', 'Stradona', and 'Zi' Francesco'.

Young leaves were collected at the Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, Università di Napoli Federico II, or at the Azienda Sperimentale Improsta (Eboli), Consorzio per la Ricerca Applicata in Agricoltura, Regione Campania.

To test the genetic uniformity of trees cultivated in or around Mt. Vesuvius, leaves from the cultivars, 'Ceccona', 'Pellecchiella', and 'Portici' were harvested on different farms in the area described in the "Albicocca Vesuviana" PGI regulation.

DNA isolation, SSR analysis, and data collection

Genomic DNA isolation from young leaves and polymerase chain reaction (PCR) amplifications were carried out as described (Corrado *et al.*, 2009; Rao *et al.*, 2006). To genotype the plants, we used one primer pair (UDP97-402) developed in peach (Testolin *et al.*, 2000) and seven primer pairs developed in apricot, UDAp407, UDAp410, UDAp411, and UDAp420 (Messina *et al.*, 2004), and UDAp480, and UDAp446 (R. Testolin, unpublished; Table I).

PCR reactions (20 µl) were performed using the following conditions: 5 min denaturation at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, 30 s at the annealing temperature listed in Table I, and 1 min of elongation at 72°C. The last cycle was followed by a final incubation for 5 min at 72°C. The amplification products were resolved using an ABI PRISM 3100 Avant (Applied Biosystems, Milan, Italy) and allele sizes were calculated using GeneScan 3.7 software (Applied Biosystems), as described (Caramante *et al.*, 2009).

AFLP analysis and band scoring

AFLP assay was performed using the AFLP analysis system-I (Invitrogen Life Technologies, Milan, Italy) with some modifications. Genomic DNA (125 ng) was digested with *Eco* RI and *Mse* I for 2 h at 37°C and the restriction enzymes were inactivated at 72°C for 15 min. The DNA fragments were ligated for 20 h to *Eco* RI and *Mse* I adapters in Adapter ligation solution using T4 DNA ligase (Invitrogen Life Technologies) according to the manufacturer's instructions. For pre-selective amplification, 2.5 µl of a 10-fold diluted ligation mixture was amplified by 20 cycles of 94°C for 30 s, 56°C for 60 s, 72°C for 60 s using the *Eco* RI and *Mse* I pre-amplification primers.

For selective amplifications, 5 µl of a 30-fold diluted pre-amplified DNA reaction was used as template in a 20 µl reaction with the *Mse* I selective primers in Table II and the *Eco*-ACT primer, 5'-end labelled with hexa-chloro-6-carboxyfluorescein (HEX). PCR reactions included one cycle of 94°C for 30 s, 65°C for 30 s, 72°C for 60 s, 12 touch-down PCR cycles lowering the annealing temperature by 0.7 °C per cycle, and 23 cycles of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s. An appropriate dilution of the selective amplification reaction was added to 0.5 µl GeneScan-400 ROX size standard (Applied Biosystems) and Hi-Di Formamide (Applied Biosystems) to a final volume of 10 µl. Prior to capillary electrophoresis in an ABI PRISM 3730 (Applied Biosystems), samples were heated for 5 min at 94°C and chilled on ice. Fragment sizes, interpolated to the internal lane standard according to the Local Southern algorithm, and peak intensities, were calculated using Peak Scanner Version 1.0 software (Applied Biosystems). Only those fragments between 50 – 450 bp were used for scoring. We considered those fragments that fulfilled the default quality requirements of the Peak Scanner software for AFLPs, in duplicate PCR experiments, to be reproducible.

TABLE I
List of the SSR loci analysed in this study and their main features

SSR locus	Core	Primer sequence (5' - 3')	Ta (°C)	Allelic size range (bp)	Reference
UDAp407	$(TC)_8TT(TC)_9$	TTCTGCTACTTACAATCGTGTTCTC† AGAGCACCAGGTCTTTCTGG	59	169–216	Messina *et al.*, 2004
UDAp410	$(AG)_{24}$	TTGTTGACAAGAAGAAAACAAAGC† CAACGGGTTGGTTTCAGAAG	56	117–177	Messina *et al.*, 2004
UDAp411	$(GA)_{26}$	TCGGTGGAGAAAGAGACTGG† GTCCCCCACCCTTTACAATG	56	78–104	Messina *et al.*, 2004
UDAp420	$(CT)_{20}$	TTCCTTGCTTCCCTTCATTG* CCCAGAACTTGATTCTGACCA	56	158–178	Messina *et al.*, 2004
UDAp480	$(GA)_{30}$	GGTTCAACCAGACCAGCATT* TGGTTTGGTAGTTGATCATTGG	56	121–181	unpublished sequence
UDAp446	$(GA)_{26}$	CCTCCCCCTAGATTTTCAGC† CGTGCTTGGGACATAGATCA	54	121–179	unpublished sequence
UDP97-402	$(AG)_{17}$	TCCCATAACCAAAAAAAACACC* TGGAGAAGGGTGGGTACTTG	54	118–140	Testolin *et al.*, 2000

Ta, annealing temperature.
†5'-end-labelled with 6-carboxyfluorescein (FAM) dye.
*5'-end-labelled with hexa-chloro-6-carboxyfluorescein (HEX) dye.

Data analysis

Cultivars that showed a single amplified SSR allele were considered, by convention, to be homozygous at that locus. For each SSR, we calculated the number of effective alleles, the observed heterozygosity, the polymorphic information content, the unbiased expected heterozygosity, the fixation index, the probability of identity, and the estimated frequency of null alleles as reported by Corrado *et al.* (2009).

Genetic similarity (GS) between genotype *i* and *j* was calculated using the Jaccard coefficient:

$$GS_{ij} = a / (n - d)$$

where *a* was the number of matching amplified DNA fragments, *n* was the total number of fragments, and *d* was the number of matching absent fragments.

A dendrogram was constructed using the UPGMA algorithm, supporting nodes with a bootstrap re-sampling of 1,000 cycles.

For each AFLP primer pair, we calculated the average percentage of reproducible fragments per sample. Only reproducible fragments were used to determine: i) the percentage of polymorphic loci found in a reaction; ii) the number of effective alleles (*Ne*), where $Ne = 1 / (p_i^2 + q_i^2)$; and iii) Nei's average gene diversity per locus (*H*), which, for an AFLP marker, is equivalent to the average expected heterozygosity *He* (Nei, 1973); and (iv) the polymorphic index content (PIC):

$$PIC = [\Sigma(1 - p_i^2 - q_i^2)] / n$$

where p_i and q_i were the frequencies of the presence and absence of an allele at locus *i*, respectively, and *n* was the number of loci examined

RESULTS

Polymorphism and cultivar variability

Molecular fingerprinting was carried out using primers designed to amplify six apricot SSRs and one transferable SSR of peach (Messina *et al.*, 2004; Testolin *et al.*, 2000), that detected polymorphisms at all loci. A total of 56 different alleles were scored, with an average of 8.00 ± 0.96 (mean ± SE) alleles per locus. The effective number of alleles (approx. 60% of the total), which enabled comparisons of allelic diversity to be made across loci, independent of the allele frequency distribution, indicated that all loci were sufficiently diverse in the population analysed. This was also supported by the high PIC values which, on average, was 0.78 ± 0.06 (mean ± SD). The average allele frequency per locus was 13.6 ± 4.2% (mean ± SD), but varied greatly, ranging from 43.8% for the 130 bp allele at the UDP97-402 locus to a minimum of 1.4% for five alleles (two at the UDAP-410 and the UDAp420 loci and one at the UDAp411 locus). While, as expected, the number of alleles was negatively correlated to the average allele frequency ($r = -0.95$; $P < 0.01$), the effective number of alleles was only weakly correlated to the average allele frequency ($r = 0.67$; $P = 0.21$). Overall, the data indicated the presence of a good level of polymorphism in the analysed apricot population, essentially due to the high number of alleles and to the frequency of heterozygous loci. The genetic indices also suggested the presence of locus-specific differences. The Fixation Index was substantially positive only for the UDAp411 and UDAp480 loci, pointing to the possible presence of undetected null alleles. Estimations of null allele frequency, from the heterozygote deficiency, indicated a significant probability, higher for the UDAp411 locus. This was consistent with the very low value of *Ho*, which was essentially due to the lower number of alleles detected at this locus (Table III).

DNA samples from each pair of trees of the same cultivar showed the same SSR profile, with two exceptions. The first was 'Bocuccia Spinosa'. At the UDAp480 locus, one tree had a single peak (143 bp), while the second tree was heterozygous (143 bp and 181 bp). Genetic analysis favoured the possibility that such a difference was due to the presence of a null allele, rather than a homozygous locus. The second discrepancy was found for one tree labelled 'Bocuccia Liscia'. At the seven SSR loci, this tree had an allelic profile identical to 'Fracasso'. Therefore, it could be considered to be a case of mis-labelling or incorrect denomination.

The genetic relationships of the apricot biotypes were analysed using a distance-based hierarchical classification. The UPGMA cluster is shown in Figure 1. The dendrogram indicated the presence of a group with highly similar genotypes. This group, which originated at the node with a bootstrap value of 99 (Figure 1), was

TABLE II

Degree of polymorphism and information content for four AFLP primer combinations used to distinguish four apricot genotypes with identical SSR profiles

Selective primer	AR	Analysed bands	MR	PIC	*Ne*
Mse I-GAG	66.1%	35	34.2	0.14	1.24
Mse I-CAT	80.3%	71	46.5	0.19	1.32
Mse I-CTA	86.7%	113	39.2	0.17	1.28
Mse I-CTT	60.2%	64	40.6	0.17	1.27

AR, average percentage of reproducible bands. MR, multiplex ratio. PIC, polymorphic index content. *Ne*, number of effective alleles.

TABLE III

Genetic indices of the apricot genotypes relative to the SSR loci analysed

SSR	*Na*	AAL	*Ne*	*Ho*	PIC	*UHe*	F	EFNA	PI
UDAp407	9	0.11	5.75	0.94	0.82	0.84	–0.14	–0.66	0.09
UDAp410	10	0.10	6.56	0.86	0.84	0.86	–0.01	–0.01	0.07
UDAp411	6	0.20	3.43	0.19	0.71	0.72	0.72	0.31	0.22
UDAp420	8	0.12	5.54	0.78	0.82	0.83	0.05	0.02	0.11
UDAp480	6	0.14	5.12	0.47	0.80	0.82	0.41	0.17	0.12
UDAp446	12	0.07	4.98	0.77	0.80	0.81	0.03	0.01	0.12
UDP97-402	5	0.20	3.16	0.62	0.68	0.70	0.08	0.03	0.26

Na, number of alleles. AAL, average allele frequency, *Ne*, number of effective allele. *Ho*, observed heterozygosity. PIC, polymorphic index content. *UHe*, unbiased *He*. F, fixation index. EFNA, estimated frequency of null alleles. PI, probability of identity.

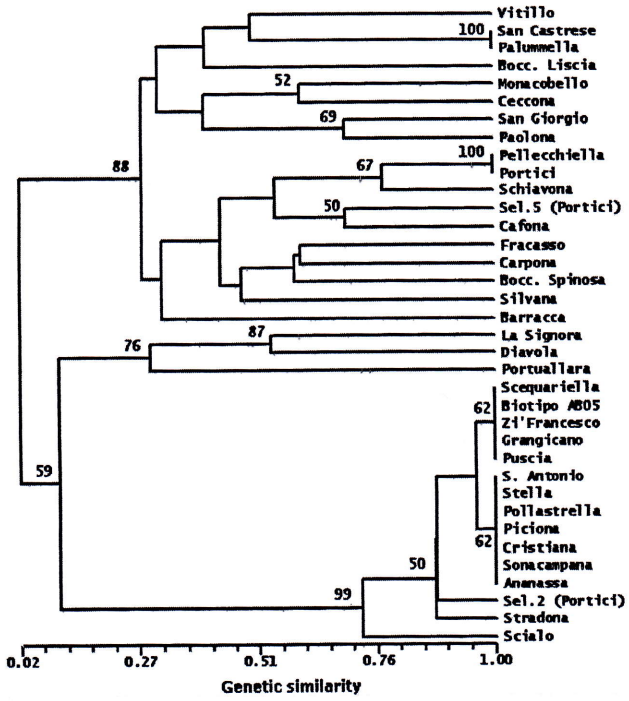

FIG. 1
Dendrogram (UPGMA algorithm) of 36 Vesuvian apricot genotypes based on genetic distances calculated using SSR data. Bootstrap values supporting nodes are shown when ≥ 50.

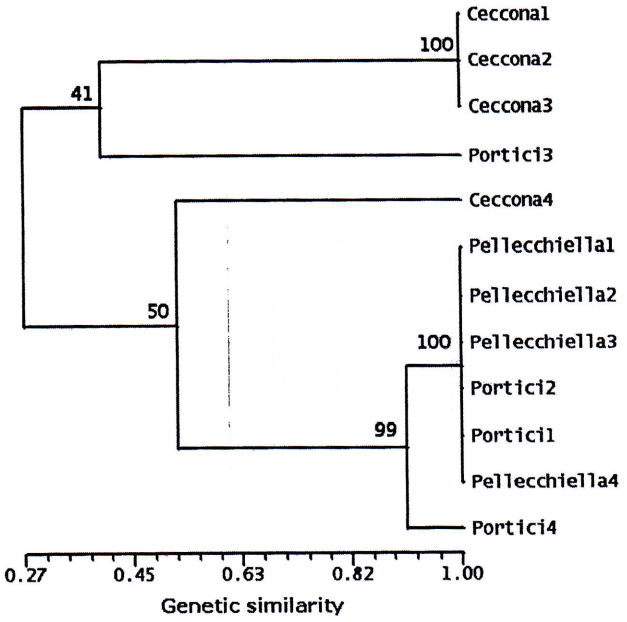

FIG. 2
Dendrogram (UPGMA algorithm) based on genetic distances calculated using SSR data from individual trees (numbered 1 to 4) of the apricot cultivars 'Pellecchiella', 'Portici' and 'Ceccona' . In each case, plant 1 was the reference tree at the "Istituto Sperimentale della Frutticoltura". Plants 2 to 4 were located on different farms of the "Albicocca Vesuviana" PGI area. Bootstrap values supporting each node are shown when ≥ 50.

characterised by having identical SSR profiles at four loci (UDAp407, UDAp410, UDAp411, and UDAp420). None of these trees were included in the "Albicocca Vesuviana" PGI regulation. In this group, 'Sel. 2 (Portici)' and 'Stradona' were the only ones that displayed a single amplification product at one locus, (UDAp480 and UDAp499, respectively), while the remaining biotypes had the same heterozygous allelic profile (121 bp and 153 bp at the UDAp480, and 129 bp and 165 bp at the UDAp446 loci). These plants were divided in two clusters, since some of them were heterozygotes (e.g., 'Ananassa') and others had a single amplification product at the UDP97-402 locus (e.g., 'Grangicano').

Among the 11 PGI cultivars, two pairs of cultivars were found to have identical SSR profiles at all loci ('San Castrese' and 'Palumella'; and 'Pellechiella' and 'Portici').

For these reasons, we extended the genetic analysis of these four PGI genotypes using AFLPs (Table II). Although related to a reduced numbers of genotypes, the best high-quality profiles (i.e., well-separated peaks, a high signal-to-noise ratio, a lack of shoulder or stutter peaks, and fragments distributed throughout the available size range) were found for the *Mse* I-CTA and *Eco* RI-ACT primer combination. Each of the four primer pairs was polymorphic, but they could not distinguish between 'San Castrese' and 'Palumella', or between 'Pellechiella' and 'Portici'. To investigate the high degree of similarity between these genotypes further, we compared the reference trees at the "Istituto Sperimentale per la Frutticoltura" to trees growing on farms of the 'Albicocca Vesuviana' PGI area. This assay also provided a clue about intra-cultivar variability. This analysis included three plants of 'Pellecchiella' and three of 'Portici', and also trees of a PGI cultivar ('Ceccona') with a unique SSR profile. Figure 2 shows the UPGMA dendrogram based on genetic distances calculated from the SSR data. The data confirmed that 'Pellecchiella' and 'Portici' had an identical SSR profile. Furthermore, 'Portici' sample 4 had a different profile at the UDAp410 locus compared to the reference tree and the other two samples. Finally, SSR fingerprints also indicated the presence of incorrect denominations on farms (i.e., 'Ceccona' 4 and 'Portici' 3).

DISCUSSION

A molecular analysis of Vesuvian PGI apricots using SSRs indicated slightly lower values of alleles detected and observed heterozygosity than those reported by Messina *et al.* (2004) and by Testolin *et al.* (2000), yet similar to the values reported in other studies (Hormaza, 2002; Romero *et al.*, 2003; Zhebentyayeva *et al.*, 2003). The average Fixation Index of our samples (a measure of population differentiation based on genetic polymorphism data) showed a lower value when compared to that reported by Romero *et al.* (2003), but similar to that obtained by analysing Tunisian apricots (Khadari *et al.*, 2006). Taking into account the fact that the trees analysed in the present study were cultivated in a limited geographic area, the data indicated a significant level of polymorphism and the presence of a potentially interesting level of heterozygosity among the Vesuvian apricot varieties. This can be explained by taking several different factors into consideration: a bias towards polymorphic SSR loci, the low selection pressure applied by breeders to Vesuvius apricots over the past decades, and because the introduction of (and replacement with) new genotypes is rare (Forlani and Pugliano, 1997). Furthermore, cultivation of apricot in the Vesuvian area

is widely based on traditional farming, causing an appreciable elevated varietal promiscuity (Forlani and Pugliano, 1997).

In order to estimate the genetic relationships among samples, we performed a UPGMA hierarchical classification based upon the genetic distance matrix, which allocated the cultivars into well separated branches. One of these included genetically similar genotypes that were not included in the PGI regulation due to their limited importance and wider distribution. We cannot exclude the possibility that these biotypes were derived from a single entity, and that the very low level of genetic polymorphism observed was due to intra-cultivar variability. Members of this group may have different denominations because, along with the traditional varieties, the cultivation of apricot is also based on shared material that is usually named after local customs (Forlani and Pugliano, 1997).

SSR fingerprints indicated that 'Portici' and 'Pellecchiella', and 'San Casterese' and 'Palummella,' were identical at the seven loci analysed. 'San Casterese' and 'Palummella' shared numerous morphological descriptors (Della Strada *et al.*, 1989). On the other hand, 'Portici' and 'Pellecchiella' were described as different varieties (Della Strada *et al.*, 1989); thus, the SSR data strongly suggest that if so, these two cultivars should be very closely-related.

A similar number of microsatellites (SSR markers) to those we used is generally sufficient to discriminate between even a high number of tree varieties (Krichen *et al.*, 2006; Sarri *et al.*, 2006; Zehdi *et al.*, 2004). Nonetheless, we analysed these suspected cases of genetic synonymy using another highly informative DNA marker system (AFLP) which can detect intra-varietal differences (Rao *et al.*, 2009). The data clearly confirmed that 'Portici' and 'Pellecchiella' (and 'San Castrese' and 'Palumella') were most identical, although it is not possible to fully exclude the presence of sport mutations.

In peach, SSRs and AFLPs could distinguish many, but not all known, sport mutations (Testolin *et al.*, 2000; Zhebentyayeva *et al.*, 2003). To exclude cases of mis-labelling or sampling errors, we extended the AFLP analysis to trees growing in the PGI area. The data further confirmed the genetic identity of 'Portici' and 'Pellechiella' and, as expected, also revealed cases of incorrect denomination (Caramante *et al.*, 2009; Rao *et al.*, 2006). In the future, it will be interesting to complement such molecular analysis with pomological and phenotypic descriptions, to assess possible differences between these PGI cultivars.

In conclusion, the results of the present study indicated the presence of good genetic differentiation in the Vesuvian apricot population, and confirmed that DNA molecular markers are a powerful tool to reveal the presence of genetic inconsistencies. Furthermore, we could identify "Albicocca Vesuviana"-specific profiles that can be used to improve the genetic traceability of PGI-labelled products (Melchiade *et al.*, 2007).

Autochthonous animals and plants, specifically adapted to local environments, are the basic resource to make a product "typical". This important level of biodiversity can be preserved by promoting the economic sustainability of traditional production systems by adequate legal protection, which in turn will also encourage the economic stability of populations in rural areas. Thus, our work not only confirmed that DNA molecular markers are a necessary tool with which to protect and distinguish premium products (Melchiade *et al.*, 2007; Rao *et al.*, 2006), but also indicated that they should be deployed during the institution of new designations of traditional materials. Finally, this work also confirms that the protection of specific products by regional institutions should include on-farm molecular investigations before and after the institution of new labels.

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