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Local and systemic gene expression of sesquiterpene phytoalexin biosynthetic enzymes in plant leaves

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Abstract Production of antimicrobial metabolites known as phytoalexins is considered as one of the initial and main barriers to inhibit pathogen development in local infected aerial tissues. Capsidiol is the main bicyclic sesquiterpene phytoalexin in tobacco (*Nicotiana tabacum*) and chili pepper (*Capsicum annuum*). Production of 5-*epi*-aristolochene by the corresponding sesquiterpene cyclase enzymes is considered the critical step in capsidiol biosynthesis. To analyze the transcriptional activation of chili pepper 5-*epi*-aristolochene synthase gene expression in response to several pathogen-associated molecular patterns, a 1,455 bp promoter fragment upstream start codon was fully sequenced and fused to β-glucuronidase reporter gene. Analyses of spatial and temporal patterns of hybrid gene expression were carried out in transgenic tobacco plants. Surprisingly β-glucuronidase was detected in both, the locally treated and the phylotactically adjacent leaves. A particular systemic gene expression was localized in the immediate vascular tissue. The activation patterns of 5-*epi*-aristolochene synthase transcripts and detection of capsidiol in corresponding tobacco and pepper systemic leaves confirmed these results. This expression pattern might be mediated by reactive oxygen species. This is the first report of a highly localized systemic gene expression of enzymes directly involved in sesquiterpene phytoalexin biosynthesis in leaves, elicited by pathogen-associated molecular patterns.

Keywords *Capsicum annuum* · β-glucuronidase reporter gene · *Nicotiana tabacum* · Pathogen-associated molecular patterns · Pepper 5-*epi*-aristolochene synthase gene · Reactive oxygen species

Abbreviations

CFU	colony-forming units
DAB	3, 3′-diaminobenzidine
FPP	farnesyl diphosphate
GUS	β-glucuronidase reporter gene
HMGR	3-hydroxy-3-metylglutaryl CoA reductase
HR	hypersensitive response
JA	jasmonic acid
MAPK	mitogen-activated protein kinase
PAMPs	pathogen-associated molecular patterns
PCD	programmed cell death
PEAS	5-*epi*-aristolochene synthase gene
PR	pathogenesis-related proteins
ROS	reactive oxygen species
SAR	systemic acquired resistance

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TEAS	tobacco 5-*epi*-aristolochene synthase gene
UBC2	ubiquitin conjugating enzyme gene
PAL	phenylalanine ammonia lyase

Introduction

Plants recognize numerous and highly conserved pathogen-derived molecules referred to as Pathogen-Associated Molecular Patterns (PAMPs) (Nürnberger and Brunner 2002). This recognition triggers numerous responses, initially at the place where the pathogen contacted the plant cell and systemically later on to protect the whole plant. Recognized local responses involve changes in ion fluxes, production of reactive oxygen species (ROS), mitogen-activated protein kinase (MAPK) activity, and expression of a large number of defence genes encoding pathogenesis-related (PR) proteins or enzymes involved in biosynthesis of antimicrobial compounds. In addition, the generation of hypersensitive response (HR), a form of programmed cell death (PCD), has been observed at the infection site that inhibits pathogen development (Garcia-Brugger et al. 2006). Another inducible event during plant pathogen interaction is the establishment of systemic acquired resistance (SAR), a long-lasting and broad-spectrum defence response throughout the plant where salicylic acid (SA) is involved. ROS and SA are related in the biosynthesis of mobile signals, which could mediate systemic expression of *PR* genes and those involved in redox regulation in other tissues (Grant and Lamb 2006).

Phytoalexin production is considered to be among initial barriers against pathogens in locally infected tissues. Biosynthesis of phytoalexins at inhibitory concentrations is a defence response highly associated with resistance, and relies on the correct location and time-course of phytoalexin biosynthetic enzyme activities (Hammerschmidt 1999). The bicyclic sesquiterpene capsidiol is the main phytoalexin in tobacco (*Nicotiana tabacum*) and chili pepper (*Capsicum annuum*). In both plants, the channelling of farnesyl diphosphate (FPP) from cytosol to 5-*epi*-aristolochene production by sesquiterpene cyclase enzyme activity is considered as the committed step in this biosynthesis. Then, 5-*epi*-aristolochene is dihydroxylated later on to produce capsidiol (Takahashi et al. 2005). Gene families encoding sesquiterpene cyclases including those involved in capsidiol biosynthesis have been demonstrated in both tobacco and chili pepper.

Among 12 to 15 copies of the 5-*epi*-aristolochene synthase gene have been detected in tobacco by Southern blot, but only *TEAS4* has been previously characterized as elicitor-inducible. This gene responds to *Pseudomonas syringae* and fungal derived elicitors, showing highly localized gene expression at the inoculation site (Yin et al. 1997). Also, elicitors from *Phytophthora* and algal polysaccharides induced *TEAS* gene expression in both the infiltration zone and the surrounding tissue in treated leaves (Ghannam et al. 2005). In our workgroup, five to eight members of pepper sesquiterpene cyclase gene family have been demonstrated. A genomic clone harbouring complete *PEAS1* gene was isolated, sequenced and respective gene expression was shown in plants challenged with *Phytophthora capsici* (Zavala-Páramo et al. 2000). Two other pepper genes (*CASC1* and *CASC2*) isolated from UV irradiated leaves, revealed 5-*epi*-aristolochene synthase enzyme activities in the *E. coli* heterologous gene expression system (Back et al. 2000). The equivalent biochemical function of *TEAS4* and *PEAS1* enzymes, suggest similarities in particular signal recognition or molecular mechanisms driving respective gene expression.

To study the molecular control of the specific *PEAS1* gene expression, a 1,455 bp promoter fragment upstream start codon of this gene was fully sequenced and fused to GUS reporter gene. Full nucleotide sequence of *PEAS1* including the promoter region here described has the GenBank No. AJ005588. In this work, we present the results of the analysis of this construction, promoter *PEAS1*-GUS gene, in transgenic tobacco plants after treatment with different elicitors. Cellulase from *Trichoderma viride* was used as a general elicitor and Pep-13 as a specific elicitor for tobacco. Elicitins are a family of small proteins secreted by species of *Phytophthora* and they are thought to be major determinants of the resistance response of tobacco against pathogens. Pep-13 is a 13 amino acid peptide derived from a 42 kDa cell wall glycoprotein of *Phytophthora* species and can induce vigorous defence responses in tobacco (i.e., hypersensitive cell death and resistance against subsequent pathogen attack) (Yu 1995). Defence responses that are ROS-dependent or ROS-independent have been identified in parsley after Pep-

13 treatment (Brunner et al. 2002; Lee et al. 2004) and this peptide-induced oxidative burst and other typical defence responses in potato (Halim et al. 2004). Previously, we reported the *PEAS1* gene expression in pepper stems and roots after infection of *P. capsici* (Zavala-Páramo et al. 2000) suggesting that this gene could, somehow recognize molecules derived from *Phytophthora*. So, Pep-13 was tested as PAMP in our transgenic plants to check the *PEAS1* promoter response. Unexpectedly, a ROS-related gene expression of *PEAS* and *TEAS* genes as well as phytoalexin accumulation were observed in corresponding systemic tissues.

Materials and methods

Plant growth conditions

Chili pepper (*Capsicum annuum*, cv. San Luis) and tobacco plants (*Nicotiana tabacum* cv. *Xanthi*) were raised in half strength MS with gelrite (2.5 g l^{-1}) in a growth chamber at 28°C with 16 h light and 8 dark photoperiod. The third older leaf of plants with six leaves was treated by infiltration with aqueous elicitor or mock solutions using a syringe without a needle. After treatments the whole plant was incubated for different times, and then the infiltrated (local) and the phylotactically adjacent fourth older leaf (systemic) were harvested at different times and frozen in liquid N_2 until use. *Agrobacterium tumefaciens* strain EHA105 was used to generate transgenic tobacco plants using standardized protocols (Horsch and Klee 1986).

PAMP and bacterial treatments

PAMPs used were Pep-13 (100 μM) (Kindly donated by Dr. Dierk Scheel from Stress und Entwicklungsbiologie, Leibniz Institut für Pflanzenbiochemie, Hälle, Germany) and cellulase (0.5 mg ml^{-1}) from *T. viride* (Sigma) in deionized sterile water; mock was the same water only. Also *P. syringae* pv. *syringae* 3525 and *P. syringae* pv. *tabaci* PTBR2004 (Kindly donated by Dr. Alejandro Peñaloza from Department of Entomology and Plant Pathology, Oklahoma State University, OK, USA) were grown overnight in King's B medium at 28°C and used to prepare a 1:100 dilution in fresh King's B medium. Bacterial suspension (10^7 CFU ml^{-1} in $MgCl_2$ 10 mM) and $MgCl_2$ 10 mM mock were infiltrated as described. Treated leaves were collected after 16 and 48 h, and used for local or systemic GUS staining respectively with X-gluc (Jefferson et al. 1987).

ROS detection by DAB uptake

Hydrogen peroxide accumulation was performed according to previous reports (Thordal-Christensen et al. 1997). Briefly, leaves were treated with 3, 3′-diaminobenzidine (DAB) (1 mg ml^{-1}) in MES 10 mM pH 3.8 and stored at room temperature for 8 h. Then leaves were cleared in boiling ethanol 96% to visualize the reddish-brown colouration.

Capsidiol extraction and quantification

Leaves were ground with 50% aqueous methanol solution and crude extracts were mixed with the same volume of ethylic ether; the organic phase was collected and dried. Residue was suspended in ethyl acetate and capsidiol was detected by Gas Chromatography (GC) performed with 1 μl aliquots on a Hewlett-Packard 5890 series II™ gas chromatograph, equipped with a HP-1 column and HP/MSD 5972 detector. N_2 was the carrier gas at a flow rate of 1.2 ml min^{-1}. Injector temperature was 230°C at 8.75 psi. After an isotherm step at 160°C for 5 min, temperature was further increased to 310°C (1°C min^{-1}). Capsidiol quantification was determined from a standard curve of pure capsidiol.

Genetic constructions

A *Bam*HI/*Hin*dIII fragment containing 1,455 bp upstream start codon and 774 bp coding region from *PEAS1* gene, was cloned into pBluescript KS+. The fragment was sequenced (ABI PRISM 3700) and analyzed using PLACE database (http://www.dna.affrc.go.jp/PLACE/) (Higo et al. 1999). Then this fragment was cloned into pCR TOPO 2.1 plasmid using a reverse primer inside *PEAS1* non-coding region (5′-GGGGATCCGCAACAGATAATAAGAG TACT-3′ *Bam*HI site underlined) and M13 forward primer in pBluescript KS+ (5′-AACATTTTGCTGCC GGTC-3′), with 20 pmol each for PCR reaction. Denaturing, annealing and extension conditions were 94°C for 1 min, 58°C for 1 min and 72°C for 2 min

respectively during 30 cycles. Reactions were performed in 50 μl final volumes with recombinant *Taq* DNA polymerase (Invitrogen). The pBI101.3 *Bam*HI/*Eco*RI fragment containing β-glucuronidase (GUS) as reporter gene and 3′-NOS terminator nucleotide sequence was cloned into pBluescript KS+. The 1,455 bp fragment from *PEAS1* gene in pCR TOPO 2.1 was fused upstream GUS using *Bam*HI site. Clones were screened by colony hybridization with a promoter fragment probe labelled by random primer Rediprime II Kit (Amersham) with (α-^{32}P)-dCTP. Restriction analysis of positive clones was used to identify those with the right orientation. The fragment with the transcriptional fusion was released with *Xba*I/*Eco*RI and used to replace GUS gene in pBI101.3. The resulting plasmid holding the 1,455 bp promoter *PEAS1*-GUS gene-NOS terminator construction (*PEAS1*::GUS) was introduced into *A. tumefaciens* for plant transformation. Transgenic lines were screened by PCR and Southern blot using a NPTII gene fragment as probe labelled as described. Six independent lines harbouring one copy of T-DNA were used for homozygous F2 plants production where PAMPs and bacterial treatments were carried out.

Analysis of gene expression

Frozen plant leaves were grounded using TRIZOL reagent (Invitrogen) for total RNA extraction. Synthesis of first strand cDNA from 200 ng RNA was carried out with the SuperScript II Reverse Transcriptase kit (Invitrogen), according to the manufacturer's instructions. Two μl of each RT reaction was used as template for PCR in a 50 μl final volume with the recombinant *Taq* DNA Polymerase (Invitrogen), and 20 pmol of each synthetic oligonucleotide primer. Forward and reverse primers used in this experiment were: 5′-CGCAAAAGAAAGACTGAAAGAA-3′ and 5′-GCAATGCACTGCACAACTAC-3′ for *PEAS1*; 5′-GCTCAGGCAACACGGTTT-3′ and 5′-TGCCCAAAAGTAGCATTCAA-3′ for *TEAS* (GenBank No. L04680); 5′-TGATAGCGCGTGACAAAAAC-3′ and 5′-GAAGAGACTGGTGAGGGATTTTAAG-3′ for GUS (GenBank No. U12639); and 5′-GAAGAGACTGGTGAGGGATTTTAAG-3′ and 5′-GCGCACCTTCCTGTTGTATTCG-3′ for the constitutively expressed ubiquitin conjugating enzyme gene (*UBC2*) (GenBank No. AB026056). Denaturing, annealing and extension conditions were 94°C for 1 min, 61°C for 1 min and 72°C for 1 min respectively for 27 cycles. PCR products were analyzed by electrophoresis in 1% agarose gels stained with ethidium bromide.

Real-time RT-PCR was carried out in the iCycler iQ Detection System (Bio-Rad Laboratories, Hercules CA, USA) using Platinum SYBR Green qPCR Supermix UDG (Invitrogen) with fluorescein 10 nM according to supplier's recommendations. Previous to reverse transcription reaction, samples were treated with Deoxyribonuclease I by 45 min, then 200 ng total RNA was used for reverse transcription with 2 pmol each primer as follows: 5′-GCAATGCACTG CACAACTAC-3′ and 5′-GGCAAATGCTTTCG CAGTTGTTCG-3′ primers for cDNA synthesis of *PEAS1* and 18S respectively. Then 2 μl (1/10) of RT reactions were used with 10 μl of 2X Supermix and 4 pmol of each primer for 20 μl reactions. Quantification of *PEAS1* transcript was carried out with 5′-GCTGCCAATCGTTGGTCTTA-3′ and 5′ GCAATG CACTGCACAACTAC-3′ primers. For internal normalization with 18S chili pepper sequence the primers were 5′-CGGGATCGGAGTAATGATTAACAG-3′ and 5′-GGCAAATGCTTTCGCAGTTGTTCG-3′. The thermal cycling parameters were 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min. A melting curve was programmed after each run to verify the lack of primer dimerization or non-specific amplification. Maximal amplification efficiency was assumed for all samples at determined threshold in log phase with the formula $T=X2^{CT}$, where T was the selected threshold of fluorescence units, X was the initial copy number and CT was the cycle number crossing the threshold. Relative gene expression was normalized to mock samples having the lowest gene expression.

Results

Cis responsive elements in *PEAS1* promoter region

PLACE database computer analysis of the 1,455 bp *PEAS1* promoter region allowed the identification of potential *cis* regulatory elements, which could be involved in control of *PEAS1* transcription gene. There were seven W boxes (TTGAC(C/T)), which could be recognized by WRKY transcription factors

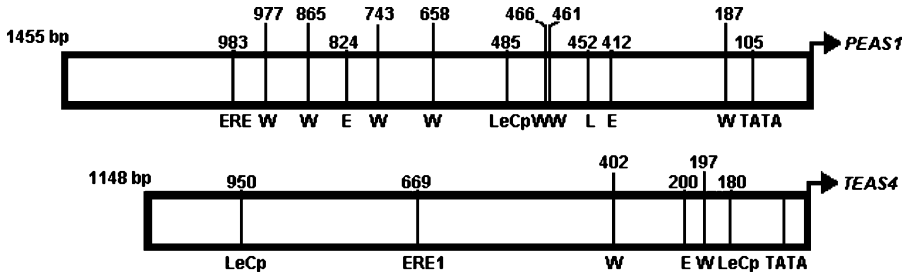


Fig. 1 Comparison of nucleotide sequence among promoter regions of *PEAS1* and *TEAS4* genes. Comparison of putative *cis* acting elements in the 1,455 bp promoter fragment of *PEAS1* and the 1,148 bp promoter fragment of *TEAS4* using PLACE database. The *number* indicates the distance from translation start site (*arrow*). *W* W boxes (TTGAC(C/T)), *ERE* ethylene response element (GCCGCC), *L* L box (TCACCTAAA), *LeCp* element recognised by the cysteine protease LeCp (TAAAA-TAT), *E* the bZIP and bHLH binding sites or E box (CANNTG), *TATA* classic TATA box

and may have a role to orchestrate changes in transcriptional patterns under pathogen attack (Ulker and Somssich 2004). Others were the ethylene response element (GCCGCC), the L box (TCACCTAAA) characterized as a UV radiation or elicitor response element, the TAAAATAT sequence found in tomato *acs2* promoter and recognized by the cysteine protease LeCp after fungal elicitor treatment (Matarasso et al. 2005) and the E box, (CANNTG) for bZIP and bHLH binding sites (Kodama and Sano 2006). Same analysis was carried out with the previously reported *TEAS4* nucleotide sequence (Yin et al. 1997) and no similarity was found between *PEAS1* and *TEAS4* promoter regions at nucleotide and potential *cis*-regulatory elements (Fig. 1).

*PEAS*1 promoter can drive different patterns of GUS gene expression

Several F2plants derived from *Agrobacterium*-mediated transgenic tobaccos holding single copies of the *PEAS1*::GUS construction, were confirmed by PCR and Southern blots (data not shown). The third older leaves from these plants were infiltrated with several PAMPs at different concentrations. After cellulase treatment, local and systemic hydrogen peroxide accumulation and *PEAS1*::GUS gene expression were detected (Fig. 2a). In systemic leaves a very peculiar *PEAS1*::GUS gene expression was shown at the adjacent parenchyma cells of primary and secondary vascular vessels (Fig. 2b). However, hydrogen perox-

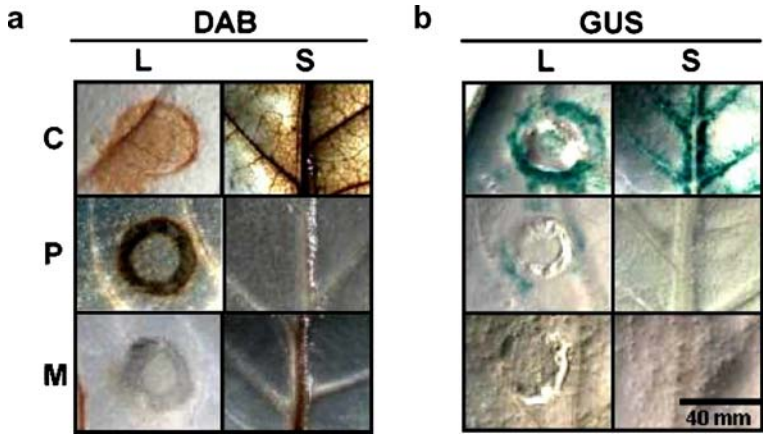


Fig. 2 ROS and GUS assays in transgenic PAMPs treated tobacco leaves. **a** DAB staining for hydrogen peroxide detection in the (*L*) local leaf after 3 h, and in the (*S*) systemic leaf after 8 h of treatments with (*C*) 0.5 μg μl^{-1} cellulase, (*P*) 100 μM Pep-13 or (*M*) mock. **b** GUS staining for *PEAS1*::GUS gene expression assay in the (*L*) local leaf after 8 h, and in the (*S*) systemic leaf after 48 h of treatment with same PAMPs. *Black bar* shows the scale proportion

ide accumulation was not restricted to these cells and was also detected in the mesophyll. Infiltration of Pep-13 elicited *PEAS1*::GUS gene expression in the surrounding edge of infiltrated zone, but we were not able to detect hydrogen peroxide accumulation suggesting that Pep-13 induction was a ROS independent mechanism. Mock inoculation did not induce *PEAS1*:: GUS expression or hydrogen peroxide accumulation in local leaves, but a weak hydrogen peroxide accumulation was detected in vascular tissue of all systemic leaves. Perhaps the infiltrated liquid increased the pressure of vascular tissues and this pressure increase is directly involved in the deposition and assembly of a new wall (Proseus and Boyer 2006). Because hydrogen peroxide is required for cross-linking reactions in lignin biosynthesis, an effect of infiltration may be the H_2O_2 detection used for the new cell wall biosynthesis (Iiyama et al. 1994).

Although other potential elicitors were used, no GUS expression was found with methyl jasmonate, SA, exogenous hydrogen peroxide or arachidonic acid. Previously, we demonstrated that arachidonic acid elicited *PEAS* gene expression in pepper plants, but tobacco did not respond to this elicitor (García-Pineda and Lozoya-Gloria 1999). Probably tobacco did not sense this acid or a different signal pathway was required. On the other hand and in our experience methyl jasmonate, salicylic acid and exogenous hydrogen peroxide did not elicit phytoalexin pathway in pepper (data not shown).

In order to elucidate if systemic *PEAS1::*GUS gene expression could be elicited by pathogens, we challenged transgenic plants with avirulent *P. syringae* pv. *syringae* 3525 and virulent *P. syringae* pv. *tabaci* PTBR2004. Local hydrogen peroxide accumulation was detected after avirulent and virulent pathogen inoculation, but systemic ROS was only detected in response to the avirulent pathogen (Fig. 3a). Local *PEAS1::*GUS gene expression was shown in response to both *Pseudomonas* strains, but systemic expression was only present in response to the avirulent strain (Fig. 3b). A transversal cut view of this systemic leaf, showed the very specific expression in parenchyma cells surrounding the vascular tissue (Fig. 3c). Again the hydrogen peroxide accumulation in vascular tissues after mock treatment may be due to lignin biosynthesis.

In order to check the elicitation effects on endogenous *TEAS* gene expression, we performed RT-PCR with specific oligonucleotides to amplify

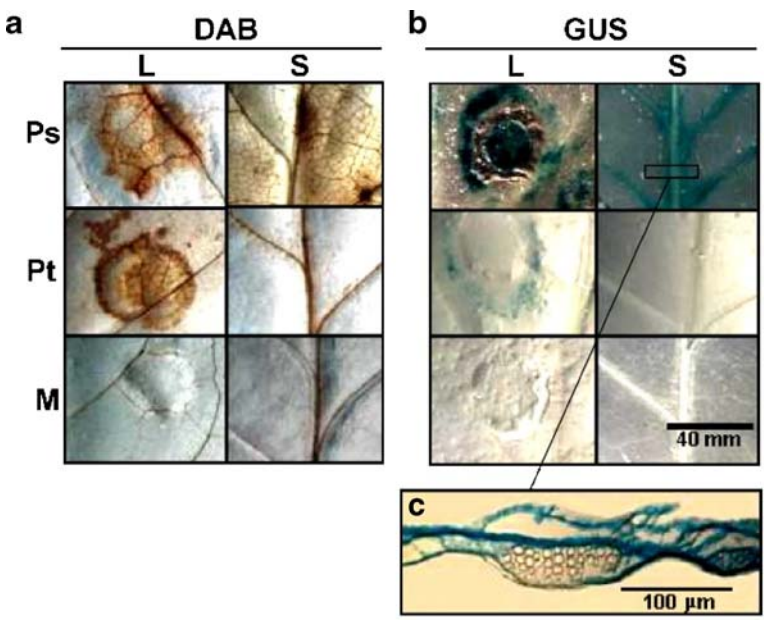


Fig. 3 ROS and GUS in transgenic tobacco leaves after pathogen treatments. **a** DAB staining for hydrogen peroxide detection in the (*L*) local leaf after 3 h, and in the (*S*) systemically infected leaf after 8 h of treatment with (*Ps*), *Pseudomonas syringae* pv. *syringae* 3525; (*Pt*), *Pseudomonas syringae* pv. *tabaci* or (*M*) mock. **b** GUS staining for *PEAS1*:: GUS gene expression assay in the (*L*) local leaf after 8 h, and in the (*S*) systemically infected leaf after 48 h of treatment with the same elicitors. *Black bars* show the scale proportions

both GUS and *TEAS* transcripts with transgenic plant RNA samples. Relatively similar results for local and systemic *PEAS1*::GUS and *TEAS* gene expression were observed after cellulase, Pep-13, and mock treatments (Fig. 4). Cellulase induced a local strong response between 4 to 8 h and similar systemic *PEAS1*::GUS and *TEAS* genes expression after 24 h of treatment. With Pep-13, local *PEAS1*::GUS gene expression persisted until 24 h after treatment, but *TEAS* had a transient gene expression pattern with a maximum at 4 h after infiltration. However, no *PEAS1*::GUS or *TEAS* transcripts were detected in systemic transgenic tobacco leaves at 0, 24 or 48 h. Whether these transcripts could be induced between 0 and 8 h is unknown. No transcripts were detected in mock treatments, *UBC*2 gene expression was constitutive and data were consistent with the histochemical GUS staining (Fig. 2).

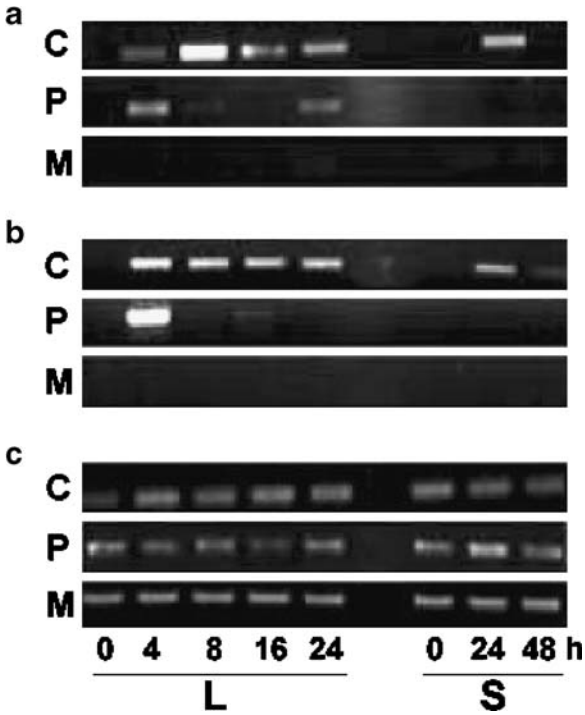


Fig. 4 Analysis of *PEAS1*::GUS and *TEAS* gene transcripts in transgenic tobacco leaves. Transcripts of **a** GUS gene driven by *PEAS1*, **b** *TEAS* and **c** *UBC*2 genes in the (*L*) local and (*S*) systemically infected leaves after indicated times of treatments with (*C*) 0.5 μg μl^{-1} cellulase, (*P*) 100 μM Pep-13 or (*M*) mock

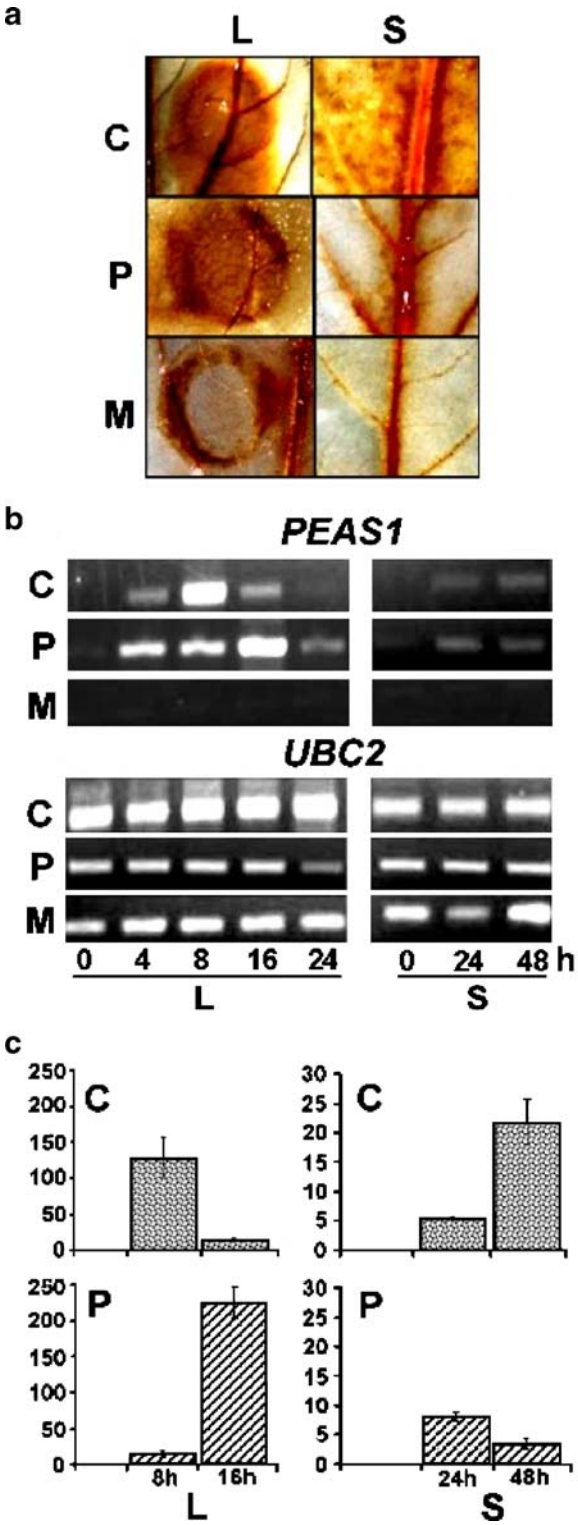
Elicitation of *PEAS1* expression

The analysis of transgenic tobacco showed a systemic *PEAS1*::GUS gene expression and endogenous *TEAS* transcript detected in these plants after the assayed stimuli. So, in order to elucidate if these expression patterns were conserved in chili pepper, we analyzed the expression of endogenous *PEAS1* in chili pepper plants in response to cellulase and Pep-13. In contrast to tobacco, hydrogen peroxide accumulated in local and systemic pepper leaves after cellulase and Pep-13 treatments (Fig. 5a). The transcript level of endogenous *PEAS1* gene was assayed by RT-PCR and quantified by real-time RT-PCR several times after infiltrations. Between 4 and 16 h after cellulase and Pep-13 treatments but not mock, a significant increase of *PEAS1* transcript was shown in local tissues. In systemic pepper leaves, we detected a light but clear increase of endogenous *PEAS1* transcript at 24 and 48 h after infiltration of cellulase and Pep-13. Mock and *UBC*2 as control of gene expression were as expected (Fig. 5b). Real-time RT-PCR showed that Pep-13 induced twice more *PEAS1* gene expression at 16 h than cellulase at 8 h after infiltration in local tissues. In systemic leaves, cellulase induced four times more *PEAS1* gene expression at 48 h than Pep-13 at 24 h (Fig. 5c).

All these data demonstrate that endogenous *PEAS1* and *TEAS* genes, both involved in sesquiterpene phytoalexin biosynthesis and traditionally considered as very specific for local responses, were found in local and systemic PAMPs elicited leaves of tobacco and pepper plants.

Capsidiol accumulation in local and systemic leaves

To confirm the role of elicited sesquiterpene cyclase gene expression in capsidiol production, it was assayed in local and systemic leaves of tobacco and pepper plants after cellulase, Pep-13 and mock treatments (Table 1). In all cases mock did not induce capsidiol production. In tobacco, the phytoalexin concentration was increased twice in cellulase treatment at local rather than at systemic leaves, and in general it was ten times less in these tissues after Pep-13 treatment. In pepper, capsidiol production was almost the same in local and systemic tissues elicited with both PAMPs. However, cellulase seemed to induce more capsidiol production in systemic leaves and Pep-13 in local leaves.



◀ **Fig. 5** Analysis of ROS and *PEAS1* gene expression in pepper leaves. **a** DAB staining for hydrogen peroxide detection in the (*L*) local leaf after 3 h, and in the (*S*) systemically infected leaf after 8 h of treatment with indicated PAMPs. **b** Transcripts of *PEAS1* and *UBC2* genes in the (*L*) local and (*S*) systemically infected leaves after indicated times of PAMPs treatment. **c** Relative *PEAS1* transcript amount measured by real time RT-PCR in the (*L*) local and (*S*) systemically infected leaves after indicated times of PAMPs treatment. PAMPs were (*C*), 0.5 μg μl^{-1} cellulase; (*P*), 100 μM Pep-13 or (*M*), mock. Each *bar* corresponds to the mean value from three assays; maximum value of each component was taken in relation to the pepper 18S transcript data. *Error bars* refer to standard error of the mean

Discussion

In previous work, transgenic tobacco plants carrying the GUS reporter gene driven by tobacco sesquiterpene cyclase *TEAS4* promoter were studied regarding the temporal and spatial expression pattern of the reporter gene. Unfortunately, assays in these transgenic plants were performed on detached leaves and systemic expression was not reported (Yin et al. 1997). In our work, nucleotide sequence comparison of promoter regions of *TEAS4* and *PEAS1* were carried out and although gene products catalyse the same step in phytoalexin biosynthesis, results showed that each one had specific arrays of *cis*-responsive elements.

Infiltration of cellulase and Pep-13 in our transgenic tobaccos holding the promoter *PEAS1*::GUS gene construction, triggered ROS-dependent and ROS-independent mechanisms required to induce *PEAS1*::GUS gene expression. With cellulase, a novel systemic expression pattern of GUS was apparently due to a ROS-dependent mechanism. However, Pep-13 elicited ROS-independent mechanisms which were required to induce *PEAS1*::GUS expression in the infiltrated zone. Similar local and systemic ROS gene expression patterns were observed when avirulent *P. syringae* pv. *syringae* was infiltrated, but not with virulent *P. syringae* pv. *tabaci*, which caused only local GUS expression. This local expression would imply capsidiol accumulation at the site of pathogen incidence and the highly localized systemic expression suggests a second phytoalexin barrier in the immediate vascular tissue that would prevent additional pathogen incidence. Capsidiol accumulation in local and systemic tobacco leaves after cellulase and Pep-13 treatments (Table 1), agreed with the *TEAS*

Table 1 Capsidiol concentration in tobacco and pepper, (*L*) local and (*S*) systemically infected leaves after 24 h of treatment with: (*C*), 0.5 μg μl^{-1} cellulase; (*P*), 100 μM Pep-13 or (*M*), mock

Plant	PAMP	L	S
N. tabacum	C	397.19	181.58
	P	25.62	48.28
	M	13.42	9.84
C. annuum	C	188.08	271.37
	P	196.18	151.13
	M	10.19	7.74

Values are in ng g^{-1} FW and represent the mean of two biological experiments.

gene transcript amount in same tissues (Fig. 4b). Although we were not able to detect *TEAS* gene transcript in systemic Pep-13 treated leaves, apparently there was some gene expression resulting in a small amount of capsidiol production. In chili pepper leaves, cellulase and Pep-13 were able to induce ROS accumulation and *PEAS1* gene expression in leaves locally and systemically. Capsidiol production in pepper leaves was also in agreement with the relative amount of *PEAS1* gene transcript assayed by real time RT-PCR (Fig. 5c). DAB staining in Figs. 2a and 5a showed that hydrogen peroxide and hence ROS production was higher in pepper than in tobacco leaves systematically after Pep-13 treatment. These results strongly suggest that ROS is related to capsidiol production, and that Pep-13 and perhaps arachidonic acid responses may have a similar ROS elicitation mechanism in pepper but different from tobacco. Systemic gene expression and ROS accumulation are common events in response to infiltration of stimuli, but we do not know if the systemic expression reported here causes phytoalexin biosynthesis at lethal concentrations.

In Pep-13 treated parsley cells, MAPK activity has been identified as the ROS independent mechanism that is required for PR gene expression (Lee et al. 2004) so, a similar mechanism could be involved to regulate *PEAS1*::GUS in tobacco. Accumulation of SA, ethylene and jasmonic acid (JA) have been reported after cellulase treatment (Martinez et al. 2001), so these molecules could regulate local *PEAS1*::GUS expression or mediate the induction and accumulation of ROS and hence the *PEAS1*::GUS gene expression in systemically infected leaves. Also, similar gene expression patterns as shown in Fig. 2 were observed for another key enzyme involved in the cytosolic isoprene biosynthetic pathway, the 3-hydroxy-3-metylglutaryl CoA reductase (HMGR), when tobacco leaves were infiltrated with a *Phytophthora megasperma*-derived glycoprotein (Dorey et al. 1997). This fact could support the proposal that metabolic flux is directed to capsidiol accumulation in a highly specific zone to block pathogen development. Other similar systemic expression patterns were reported in chili pepper for some defence-related genes (Lee and Hwang 2005), and in transgenic *Arabidopsis* holding GUS gene driven by P69B and P69C promoters from tomato P69 subtilisin-like proteases. These belong to a gene family of high complexity encoding protein isoforms of approximately 69 kDa accumulated in the apoplast. They were induced by salicylic acid and by compatible and incompatible interactions with *P. syringae* (Jordá and Vera 2000).

On the other hand, systemic production of phytoalexins has been reported in other systems mainly those where root tissues have been involved in the interaction. Systemic accumulation of phenolic phytoalexins was detected in cucumber leaves following application of the biocontrol agent *Trichoderma asperellum* to the root system (Yedidia et al. 2003). Accumulation of mRNA of phenylpropanoid pathway gene *PAL1* may support induction of phenolic phytoalexin biosynthesis in systemically infected leaves. Nevertheless, PAL catalyses the first step in phenylpropanoid metabolism required for lignin, anthocyanins, antioxidants and phytoalexins (Ritter and Shulz 2004). Then, PAL, unlike 5-*epi*-aristolochene synthase activity, may not be considered a committed step in phenolic phytoalexin biosynthesis. In the pathosystem chili pepper—*P. capsici*, some results suggested that *Trichoderma harzianum*, in contact with the root system, induced a systemic defence response against *P. capsici* in the upper part of the plant. The high concentration of capsidiol detected in *T. harzianum*-treated plants seems to be one factor, but not necessarily the main one in delaying lesion development in the stems of pepper plants inoculated with *P. capsici* (Ahmed et al. 2000). In this case, 5-*epi*-aristolochene synthase activity must be present for capsidiol accumulation in the systemic tissue. In our case, it still remains unclear if pretreatment with avirulent *Pseudomonas* or cellulase could induce lethal capsidiol concentration to prevent a second pathogen attack.

As far as we know, this is the first report of highly localised systemic gene expression of an enzyme directly involved in phytoalexin biosynthesis as well as the corresponding accumulation of respective phytoalexin in those tissues. With these results and those related to systemic phytoalexin production when root tissues are involved, a deep revision of the concepts of local and systemic defence mechanisms could deserve special attention.

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