Available online at www.sciencedirect.com
SCIENCE DIRECT®
PHYTOCHEMISTRY
Phytochemistry 65 (2004) 1343–1350
www.elsevier.com/locate/phytochem

Wound-induced *RNaseLE* expression is jasmonate and systemin independent and occurs only locally in tomato (*Lycopersicon esculentum* cv. Lukullus)

Nadine Groß [a], Claus Wasternack [b], Margret Köck [a,*]

[a] *Biocenter, Martin Luther University Halle-Wittenberg, Weinbergweg 22, D-06120 Halle, Germany*
[b] *Department of Natural Product Biotechnology, Leibniz-Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle, Germany*

Received 15 January 2004; received in revised form 8 April 2004
Available online 4 June 2004

Abstract

Tomato *RNaseLE* is induced by phosphate deficiency and wounding and may play a role in macromolecular recycling as well as wound healing. Here, we analyzed the role of jasmonate and systemin in the wound-induced *RNaseLE* activation. The rapid expression of *RNaseLE* upon wounding of leaves leading to maximal RNase activity within 10 h, appeared only locally. Jasmonic acid (JA) or its molecular mimic ethyl indanoyl isoleucine conjugate did not induce *RNaseLE* expression. Correspondingly, *RNaseLE* was expressed upon wounding of 35S::*allene oxide cyclase antisense* plants known to be JA deficient. *RNaseLE* was not expressed upon systemin treatment, but was locally expressed in the *spr1* mutant which is affected in systemin perception. In tomato plants carrying a PromLE::*uid*A construct, GUS activity could be detected upon wounding, but not following treatment with JA or systemin. The data indicate a locally acting wound-inducible systemin- and JA-independent signaling pathway for *RNaseLE* expression.

Keywords: *Lycopersicon esculentum*; Solanaceae; Ribonuclease LE; Expression analysis; Tomato wound response; Jasmonate-independent signaling; Systemin

1. Introduction

Piercing and chewing insects, as well as mechanical injury of plant tissues, induce chemical defense reactions (Walling, 2000). Among them, the formation of proteineous defense compounds such as proteinase inhibitors (PINs) is most prominent (Ryan, 2000; Kessler and Baldwin, 2002). Other wound-induced genes code for enzymes of phytoalexin synthesis and for signaling components. Among signals of the wound-response pathway, the peptide systemin and the cyclopentanone compound jasmonic acid (JA) are regarded to be key compounds at least in tomato. Four types of systemins occur in tomato. First, an 18 amino acid peptide called systemin and processed from a 200 amino acid peptide prosystemin was found (Ryan, 2000). Recently, Pearce and Ryan (2003) identified three wound-inducible hydroxyproline-rich, glycosylated peptides called hydroxyproline-rich systemins.

Although JA, another signal in the activation of *PIN* gene expression, is clearly downstream from systemin perception, it is not clear how its synthesis is initiated. Synthesis of JA which originates from α-linolenic acid (α-LeA) of chloroplast membranes includes an allene oxide cyclase (AOC)-catalyzed step (Feussner and Wasternack, 2002). In tomato, the formation of JA occurs transiently within 1 h upon local wounding or

Abbreviations: AOC, allene oxide cyclase; AOS, allene oxide synthase; PIN2, proteinase inhibitor 2; JA, jasmonic acid; AOCs, 35S::AOC*sense* plants; AOCas, 35S::AOC*antisense* plants; Eth-In, ethyl indanoyl isoleucine conjugate; GUS, β-glucuronidase; JAME, jasmonic acid methyl ester; *jai1*, jasmonate-insensitive 1 mutant; α-LeA, α-linolenic acid; OGAs, oligogalacturonides; OPDA, 12-oxo-phytodienoic acid; *spr1*, suppressor of prosystemin-mediated response 1 mutant, systemin-insensitive; *spr2*, suppressor of prosystemin-mediated response 2 mutant.

* Corresponding author. Tel.: +49-345-55-21-620; fax: +49-345-55-27-230.
E-mail address: koeck@biozentrum.uni-halle.de (M. Köck).

0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.04.036

systemin treatment (Howe et al., 1996; Stenzel et al., 2003) and leads together with ethylene (O'Donnell et al., 1996) and hydrogen peroxide (Orozco-Cardenas et al., 2001) to *PIN* expression. The systemin-dependent expression of *AOC* and the JA-dependent prosystemin gene expression (McGurl et al., 1992) are both located in vascular bundles (Jacinto et al., 1997; Narváez-Vásquez and Ryan, 2004; Hause et al., 2000), suggesting amplification in wound signaling. Consequently, JA accumulates preferentially in main veins upon wounding of tomato leaves (Stenzel et al., 2003).

The wound-induced *PIN2* expression of tomato occurs locally and systemically as known for most wound-induced genes (Strassner et al., 2002). The signal transduction pathways, however, differ at both sites. Recent grafting experiments with wild type and various mutants, impaired in JA biosynthesis (*spr2*) due to affected fatty acid desaturase FAD7 (Li et al., 2003), and JA signaling (*jai-1*) revealed that JA biosynthesis is required in the wounded tissue, but not in the systemic tissue which needs only intact JA perception (Li et al., 2002). Another mutant *spr1* is impaired in systemin perception and formation of a systemic wound signal as well as in the local *PIN2* activation (Lee and Howe, 2003).

Among the wound-induced genes of tomato, there is an *RNase* (Lers et al., 1998). There are at least three members of the RNase T2 family in tomato. Two of them, the *RNaseLX* and the *RNaseLE*, were highly expressed upon phosphate starvation, and function as RNA-specific endonucleases without base specificity releasing 2′,3′-cyclic nucleotides (Abel and Köck, 2001). The ER-resident RNaseLX is involved in PCD occurring during germination and senescence and has a role in tracheary element differentiation (Lehmann et al., 2001). In contrast, the RNaseLE is a vacuolar and extracellular enzyme in tomato cell suspension cultures (Abel and Köck, 2001). *RNaseLE* expression occurs specifically in developing phloem tissue (Köck et al., 2004). Due to the common occurrence of the wound-inducible *RNaseLE* expression and generation of systemin as well as JA in the vascular system, the question emerged whether the wound-induced *RNaseLE* expression is JA and systemin dependent. A combination of chemical, genetic and histological techniques were used to test for systemin and JA inducibility of *RNaseLE* expression. Levels and kinetics of *RNaseLE* expression were compared to those of *PIN2*, a classic JA-inducible wound response gene. *RNaseLE* expression was clearly systemin- and JA-independent and appeared only locally. The *RNaseLE* expression data suggest a new type of wound signaling.

2. Results

The wound-inducibility of *RNaseLE* expression in tomato leaves (Lers et al., 1998) prompted us to ask whether the expression occurs locally and systemically. Whereas the marker gene of the local and systemic wound response, *PIN2*, is clearly activated at both sites, *RNaseLE* mRNA accumulation occurred only locally with similar kinetic as *PIN2* (Fig. 1(a)).

RNaseLE is detectable in tomato leaves only at low level and is confined to vascular bundles (Köck et al., 2004). Consequently, time course analysis of *RNaseLE* expression in total leaf area in terms of protein level was difficult to perform. Therefore, we inspected RNase activity by an *in-gel* assay and quantitative activity tests (Figs. 1(b) and (c)). Among the different RNases detectable in extracts of cultivated tomato cells by the *in-gel* assay (Köck et al., 1995; Abel and Köck, 2001) only one, the RNaseLE, is activated upon wounding (Figs. 1(b) and 3(c)). Consequently, total RNase activity shown in Fig. 1(c) is indicative for RNaseLE activity. Both analyses revealed only a local increase in RNaseLE activity upon wounding.

In order to test whether the local wound-induced *RNaseLE* expression is mediated by systemin, leaves

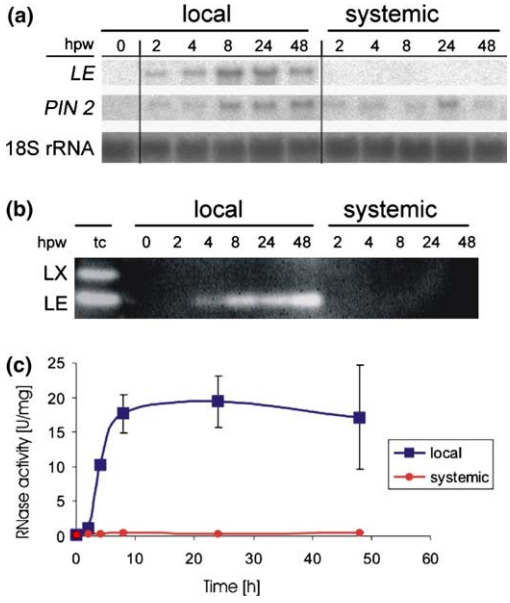

Fig. 1. The *RNaseLE* mRNA accumulation (a) and increase in RNase activity (b, c) occurs only locally. Total RNA (a) and protein (b, c) were isolated from a wounded (local) and an upper unwounded (systemic) leaf of wild type cv. Lukullus plants at the indicated time points (hpw: hours past wounding). (a) *RNaseLE* mRNA (*LE*) and *PIN2* mRNA accumulation were analyzed with corresponding radiolabeled cDNA probes. Loading of 20 μg total RNA per lane was checked by hybridizing with an 18S rRNA probe. (b) RNaseLE activity was analyzed by *in-gel* assay using 10 μg of total protein. On the left, RNase activity of total extracts from cultivated tomato cells (tc) indicates activity of RNaseLE and RNaseLX as described by Köck et al. (1995). Note that only RNaseLE activity was detected locally after wounding. (c) Total RNase activity of probes as in (b) analyzed spectrophotometrically at 260 nm. Values represent the mean of six different extractions and measurement using six plants; bars indicate the standard error.

were treated with systemin and its inactive analog systemin-17. As expected *PIN2* was activated by systemin, but not by systemin-17 treatment (Fig. 2(a)). Upon systemin treatment only residual *RNaseLE* mRNA accumulation could be detected. As expected for such a putative systemin-independent *RNaseLE* expression, *RNaseLE* mRNA accumulated upon preincubation of leaves with systemin-17 followed by wounding (Fig. 2(b)). We used also the tomato mutant *spr1* that exhibits reduced *PIN2* mRNA levels upon local wounding due to impaired systemin signaling (Lee and Howe, 2003). *RNaseLE* mRNA accumulation occurred in *spr1* leaves upon local wounding with a similar kinetic and level as in the wild type leaves (data not shown). Taken together, these data indicate systemin-independent activation of *RNaseLE* expression.

JA mediates activation of most wound-induced genes. In order to test its role in *RNaseLE* expression, we treated detached leaves with its methyl ester (JAME) as well as the highly active analog ethyl indanoyl isoleucine conjugate (Eth-In). Whereas *PIN2* mRNA accumulation appeared abundantly upon treatment with both compounds in similar kinetics, no *RNaseLE* mRNA accumulation could be detected (Fig. 3(a)). Further signals known to function in the wound response pathway were tested such as ABA, ethylene, salicylate, chitosan and oligogalacturonides (OGAs). Whereas *PIN2* mRNA accumulation appeared upon treatment with each of these compounds, no *RNaseLE* was detectable (data not shown).

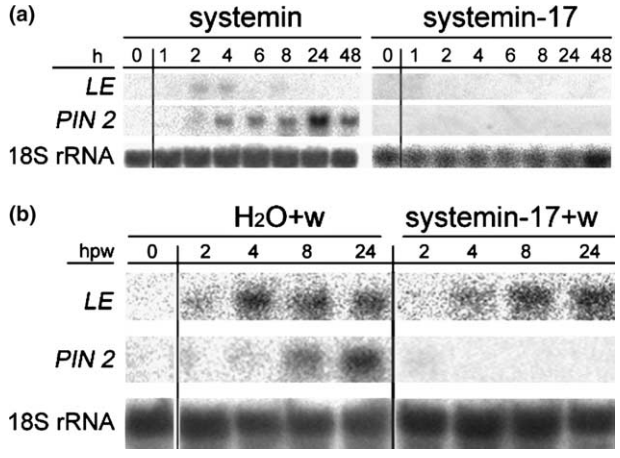

Fig. 2. The wound induced *RNaseLE* mRNA accumulation occurs systemin independent. (a) Time course of *RNaseLE* (*LE*) and *PIN2* mRNA accumulation after treatment with 5 pM systemin or 5 pM systemin-17. Freshly excised leaves of wild type cv. Lukullus plants were used and subjected to Northern blot analysis of 20 μg total RNA. Loading control was performed with an 18S rRNA probe. (b) Time course of *RNaseLE* and *PIN2* mRNA accumulation upon a 2-h pretreatment with 5 pM systemin-17 or distilled water followed by wounding (w) and flotation on distilled water, respectively (conditions as in (a)).

To test the obvious JA independence of *RNaseLE* expression, we used transgenic tomato plants overexpressing or repressing *AOC*. The AOC*sense* (AOCs) line exhibits high levels of *AOC* mRNA, AOC protein, and in vitro AOC activity as well as 2.5-fold higher JA levels upon wounding compared to wild type (Stenzel et al., 2003). In contrast, these parameters were strongly diminished in the AOC *antisense* (AOCas) plants, e.g. wound-induced JA levels reached less than 10% of the wild type level. *RNaseLE* expression occurred in both,

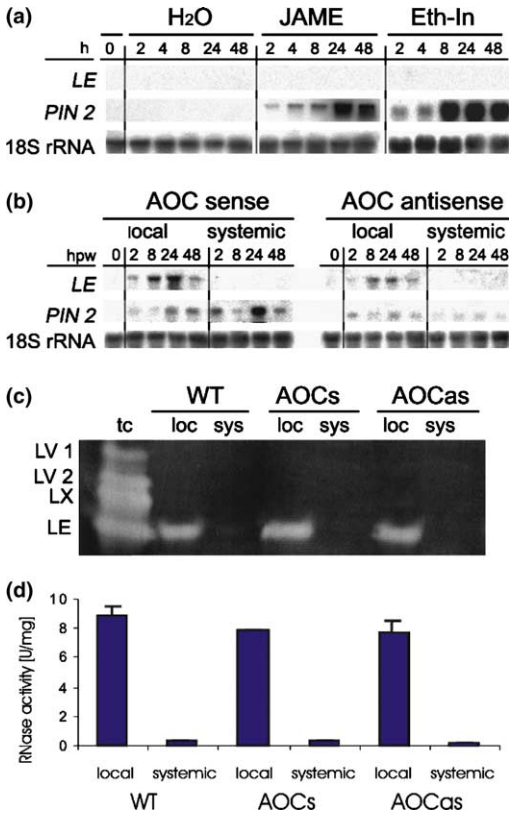

Fig. 3. *RNaseLE* accumulation occurs JA independent. (a) The *RNaseLE* and *PIN2* mRNA accumulation in response to exogenous jasmonic acid methyl ester (JAME) and ethyl indanoyl isoleucine conjugate (Eth-In). Freshly excised leaves of tomato wild type plants cv. Lukullus were floated on 50 μM JAME, 50 μM Eth-In or distilled water for indicated times. Northern blot analyses were performed with 20 μg of total RNA per lane. (b) *RNaseLE* and *PIN2* mRNA accumulation in AOCs and AOCas plants after wounding. Twenty micrograms total RNA was subjected to Northern blot analysis. (c, d) RNaseLE activity of wild type (WT) cv. Lukullus, AOCs and AOCas plants in response to wounding. Total protein was analyzed for RNase activity from wounded and unwounded (systemic) leaves of WT, AOCs and AOCas plants at 8 h after wounding. (c) RNase activity was detected by native PAGE using 10 μg total protein per lane and negative staining. The total extract from cultivated tomato cells (tc) indicates activities of RNasesLE, LX, LV2 and LV1 as described by Köck et al. (1995). Note that RNaseLE is locally induced in AOCas as well as in AOCs plants. (d) Total RNase activity was analyzed spectrophotometrically with six independent extractions and measurements from six plants. Bars indicate the standard error. Since only RNaseLE is wound induced (cf. (c)), values are indicative for RNaseLE activity. Note that this activity appeared in WT, AOCs and AOCas plants.

AOCs and AOCas plants upon wounding (Fig. 3(b)). Due to the difference in JA levels, the wound-induced *RNaseLE* mRNA accumulation in AOCas leaves indicates a JA-independent expression. In contrast, the *PIN2* mRNA appeared in AOCs plants locally and at higher levels systemically, whereas only residual *PIN2* mRNA was detected locally and systemically in AOCas plants. RNaseLE activity levels were also JA independent (Figs. 3(c) and (d)). RNase activity was locally detectable in wounded AOCas plants in both the *in-gel* assay and the quantitative activity analysis at similar levels as in AOCs plants and wounded wild type plants.

Transgenic tomato plants carrying the *uid* A gene under the control of a 2.6 kb fragment of the *RNaseLE* promoter (Köck et al., 2004) revealed GUS activity upon wounding, but only a residual level appeared after JAME or systemin treatment (Fig. 4(a)). The wound-induced JA/systemin-independent promoter activity appeared preferentially in vascular bundles (Fig. 4(b)).

3. Discussion

Since the first observation of wound-induced PIN formation in tomato leaves three decades ago (Green and Ryan, 1972), a well-established model of wound signaling exists (rev. in Ryan, 2000; Leon et al., 2001; Wasternack and Hause, 2002). Initially proposed for the wounded (local) tissue, a sequential generation and action of prosystemin → systemin → JA (ABA) → ethylene → OGAs → reactive oxygen species (ROS) was assumed to ultimately induce the expression of defense genes such as *PIN2*.

Later on a more complex scenario was built up, summarizing very early events such as membrane depolarization, proton influx, ROS generation, increase in intracellular Ca^{2+} and activation of MAP kinases followed by late gene expression. In addition to a temporal pattern in wound signaling, a spatial distribution of signals was described, indicating an amplified generation of prosystemin, systemin and JA in vascular tissues

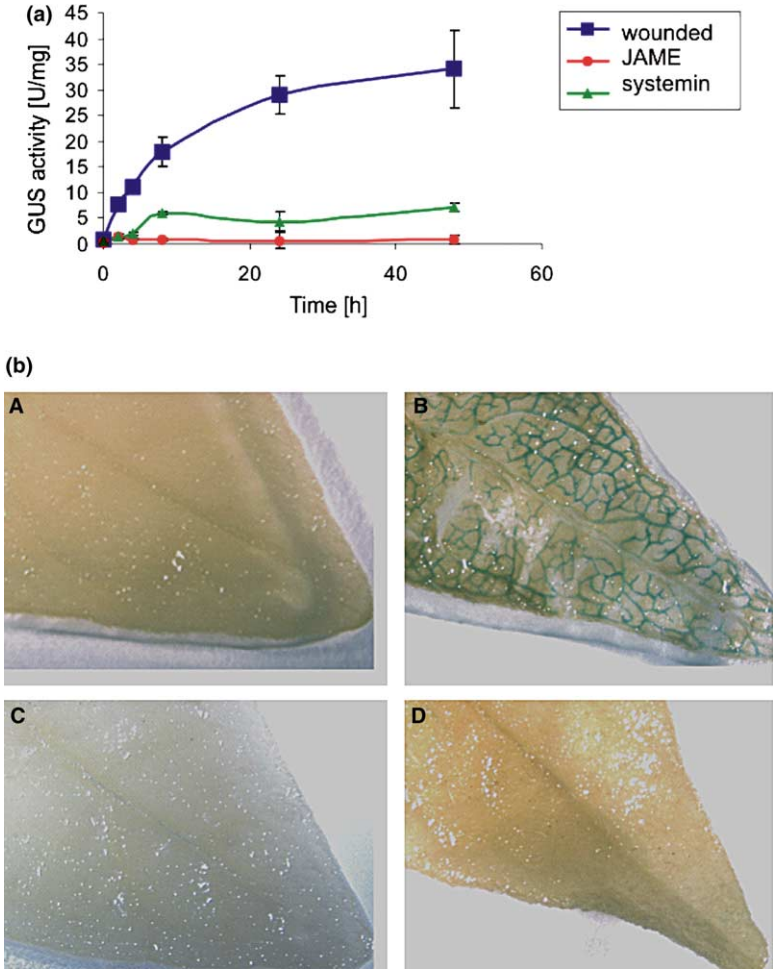

Fig. 4. The *RNaseLE* promoter is active upon wounding but not following JA or systemin treatment. (a) GUS activity in wounded leaves of six PromLE::*uid*A plants and leaves treated with 50 μM JAME or 5 pM systemin. Bars indicate the standard error ($n = 6$). (b) Histochemical localization of GUS activity in unwounded (A) and wounded (B) leaves as well as in leaves treated with 5 pM systemin (C) and 50 μM JAME (D) after 8 h. Note that the promoter of *RNaseLE* is only active in response to wounding and preferentially in the vascular system of leaves (b).

(Jacinto et al., 1997; Stenzel et al., 2003; Narváez-Vásquez and Ryan, 2004), elevation of the mobile H_2O_2 nearby (Orozco-Cardenas et al., 2001) and finally *PIN2* expression in mesophyll cells. Initially, the systemic wound response was thought to be triggered by systemin (Ryan, 2000). Recent grafting experiments, however, of wild type plants with the JA-deficient mutant *spr2*, the systemin-insensitive mutant *spr1* and the JA-insensitive mutant *jai1* suggest that JA or related compounds act as systemic signal(s) (Li et al., 2002; Lee and Howe, 2003). The accumulating data on wound responsive gene expression illustrate a greater complexity than previously recognized, e.g. (i) differences in wound-signaling between plant species (Leon et al., 2001), (ii) differences in the number and action of wound signals (Ryan, 2000; Leon et al., 2001; Wasternack and Hause, 2002), (iii) differences in the link between local and systemic responses, (iv) differences in the type of wounding caused by various insects (chewing, sucking) (Walling, 2000).

A JA-independent wound-induced gene expression occurs in *A. thaliana* (Titarenko et al., 1997) and is accompanied by an inverse action of protein phosphorylation/dephosphorylation in the JA-dependent and JA-independent signaling pathway (Rojo et al., 1998; Leon et al., 2001). Also in tomato and tobacco, both known for an essential role of JA and systemin in wound signaling, JA-independent wound-induced gene expression occurs (O'Donnell et al., 1998; Hiraga et al., 2000). *RNaseLE* of tomato is a wound-induced gene (Lers et al., 1998). During development it is specifically activated in developing phloem tissue (Köck et al., 2004). The common occurrence of *RNaseLE* expression and generation of JA as well as systemin in this tissue suggested a JA- and systemin-dependence of *RNaseLE* expression upon wounding. We show here, however, that the wound-induced *RNaseLE* expression occurs in a JA- and systemin-independent manner only locally. Pharmacological studies using treatments with systemin or its inhibitor systemin-17 followed by wounding, in wild type and PromLE*::uid*A plants reveal a systemin independence (Figs. 2(a), (b) and 4(a), (b)). This conclusion is supported by the strong *RNaseLE* mRNA accumulation in wounded leaves of the systemin-insensitive mutant *spr1*. Lee and Howe (2003) reported on the *PIN2* accumulation at similar levels in wild type and *spr1* plants following treatment with OGAs, α-LeA or JA. Only residual *PIN2* amounts, however, occurred upon treatment with systemin or recombinant prosystemin. This suggests that even in the absence of systemin perception, JA and other signals allow *PIN2* expression.

Interestingly, Lee and Howe (2003) identified other systemin-independent wound-inducible genes such as the *LOXD*, the *AOS1* and a protein kinase encoding gene (*WIPK*) using the *spr1* mutant. Wound-induced expression of these genes as well as the *RNaseLE* in the JA-deficient *def1* mutant of tomato (Howe et al., 2000) suggest that systemin-independent signaling is coupled to JA-independent signaling. The JA deficiency, however, of the *def1* mutant is partial, *def1* is not affected in the *AOS* and the *AOC* gene but exhibits reduced capacity to form OPDA (Howe et al., 1996; Stenzel et al., 2003). Therefore, we reinvestigated here a putative JA independence of *RNaseLE* expression in different approaches including: (i) treatment of leaves with JA or its analog Eth-In, a compound highly active in mimicking JA responses (Schüler et al., 2001, 2004; Stenzel et al., 2003), (ii) plants with impaired or elevated JA levels (Stenzel et al., 2003) and (iii) plants expressing GUS activity under the control of the *RNaseLE* promoter (Köck et al., 2004). All approaches support a JA-independent *RNaseLE* expression (Figs. 3 and 4).

Despite a JA- and systemin-independent expression of *LOXD*, *AOS1* and *WIPK*, all these genes are systemically activated upon local wounding (Lee and Howe, 2003). A similar expression pattern was found for *Tw1*, a tomato gene with homology to glucosyl transferases (O'Donnell et al., 1998). In *A. thaliana* where systemin(s) are absent, the RNase *RNS1*, homologous to *RNaseLE* in structure and function, is wound induced in a JA-independent manner locally and systemically (LeBrasseur et al., 2002). Whereas grafting experiments with *spr2*, *spr1*, *jai1* and wild type plants of tomato support a role of JA or related compounds in systemic signaling of JA-responsive genes (Li et al., 2002; Lee and Howe, 2003), the systemic expression of JA-independent genes such as *RNS1* of *A. thaliana* or *WIPK* of tomato might be mediated by signals formerly discussed for the JA-dependent systemic *PIN2* expression (Leon et al., 2001). Among these, signals are electric signals (Wildon et al., 1992; Stankovic and Davies, 1996) and hydraulic signals propagated through the xylem (Malone, 1996) or phloem (Rhodes et al., 1999).

The wound-induced genes can be grouped into two sets of genes: (i) Gene expression is dependent on JA and systemin and occurs both locally and systemically, e.g. *PIN2*. (ii) Gene expression is independent of JA and (systemin), but occurs locally and systemically, e.g. *WIPK*, *Tw1*, *AOS*, *LOXD* of tomato and *RNS1* of *A. thaliana*.

Here, we described a third group of genes such as *RNaseLE* which are activated upon wounding in a JA- and systemin-independent manner, but showing only local transcript accumulation and enzyme activity upon wounding in different genetic backgrounds such as wild type, AOCs, AOCas (Figs. 1–3) and *spr1* plants. Data on wound-induced GUS activity of PromLE::*uid*A plants support this (Fig. 4).

A tobacco peroxidase, *tpoxN1*, possesses a very similar expression pattern and location compared with *RNaseLE* (Hiraga et al., 2000; Sasaki et al., 2002). *TpoxN1* was rapidly expressed exclusively in vascular

bundles and epidermal cells independent from JA and other wound signals. Sasaki et al. (2002) suggest a role of POX in wound healing by facilitating suberin formation following POX-catalyzed oxidation of wound-inducible hydroxycinnamic acid derivatives.

The *RNaseLE* expression described here as a JA- and systemin independent, only locally occurring unique wound-signaling pathway and the tissue specificity of *RNaseLE* expression (Köck et al., 2004) suggest also a role in wound healing. This corresponds to the fact that further wound signals such as ethylene, ABA, OGAs, chitosan or salicylate, known to establish an increased defense status, by local and systemic expression of defense genes, do not induce *RNaseLE*. Possibly rapid expression of *RNaseLE* at the wound site may be part of an apoptotic pathway near the wound site.

4. Experimental

4.1. Plant material, growth, treatments and agents

Lycopersicon esculentum Mill. cv. Lukullus, cv. Castlemart, the mutant *spr1* and the transgenic lines (Lukullus background) carrying the full-length cDNA of the tomato AOC in *sense* and *antisense* orientation under the control of the 35S promoter (Stenzel et al., 2003) were grown at a 16 h/24 °C, 8 h/18 °C day night cycle with 300 μmol photons $m^{-2} s^{-1}$ of white incandescent light and 75% humidity. Plants used in the experiments were 4 weeks old. For treatments, leaves were cut at the petiole and floated on 50 μM JAME, 50 μM Eth-In, 5 pM systemin or 5 pM of systemin-17. Leaves were kept in petri dishes containing 50 ml per leaf of the described solutions at 22 °C under continuous white light (120 μmol $m^{-2} s^{-1}$), provided by fluorescent lamps (Narva, Berlin NC 250/01). Leaves of intact plants were wounded three times per leaflet in a distance of 2 mm using ridged flat-tipped tweezers. The wounded (local) and the upper undamaged (systemic) leaves were harvested at indicated time points. All samples were frozen in liquid N_2 immediately after harvesting and stored at −80 °C until used for RNA or protein extractions.

The *spr1* seeds were provided by G. Howe (University East Lansing, MI, USA). Systemin and systemin-17 were synthesized and provided by T. Nürnberger (University of Tübingen, Germany). The Eth–In conjugate recently described (Schüler et al., 2001) was provided by W. Boland (MPI, Jena, Germany).

4.2. Extraction of RNA and Northern blot analysis

Isolation of total RNA from tomato plants was carried out according to Köck et al. (1998). For Northern blot analysis, 20 μg total RNA per lane were loaded and analysis was performed as described (Sambrook et al., 1989). Blots were probed with [α-^{32}P]dCTP-labeled cDNA probes of *RNaseLE* (full length, Köck et al., 1995), tomato *PIN2* (800 bp fragment, Stenzel et al., 2003) and 18S rRNA probe (full length, Köck et al., 1995) as loading control. Before reuse of blots, radioactive probes were removed by stripping. Autoradiograms were analyzed with a BAS Imager 1500 (Fuji/Raytest, Straubenhardt).

4.3. Quantitative analysis of RNase activity

Ribonuclease activities were determined spectrophotometrically at 260 nm by recording of ethanol-soluble hydrolysis products released from RNA. One unit of RNase activity is defined as the amount of enzyme causing an increase in A_{260} of 1.0 $min^{-1} cm^{-1} ml^{-1}$ (Abel and Köck, 2001). Protein was assayed according to Lowry et al. (1951).

4.4. In-gel activity assay

Following homogenization of leaf tissues or cultivated tomato cells under liquid nitrogen, total proteins were extracted with 150 mM Na-acetate buffer, pH 5.6, containing 1 mM PMSF and 2 mM EDTA. Homogenates were cleared by centrifugation and soluble proteins were quantified by the method of Lowry et al. (1951). Ten micrograms of total protein was loaded on each lane. Disc gel electrophoresis on native polyacrylamide slab gels (15% acrylamide) was carried out without SDS using the buffer system according to Laemmli (1970). Detection of RNase activity was performed by washing the gels in 150 mM Na-acetate buffer (pH 5.6) for 2×10 min, followed by incubating gels in substrate solution (150 mM Na-acetate buffer, pH 5.6; 2.5 mM EDTA, 0.4% yeast RNA) at 37 °C for 30 min. After rinsing off adhering substrate solution with buffer, gels were stained in 0.2% toluidine blue, 0.5% acetic acid for 5 min and were destained in 0.5% acetic acid (Abel and Köck, 2001).

4.5. Quantitative analysis of GUS activity

Tomato leaves were ground to powder using liquid nitrogen and proteins were extracted with 100 mM phosphate buffer, pH 7.8, containing 1 mM EDTA, 7 mM 2-mercaptoethanol, 10% glycerol, and 1% Triton X-100. Homogenates were cleared by centrifugation and supernatants were used for measurement of protein content (Lowry et al., 1951) and GUS activity according to Jefferson et al. (1987).

4.6. Histochemical localization of GUS expression

Leaves were fixed in 0.3% formaldehyde in 50 mM sodium phosphate, pH 7.0, 1 mM EDTA for 30 min and

washed several times with 50 mM sodium phosphate, pH 7.0 prior to staining. Fixation and staining solutions were infiltrated under reduced pressure for 2–5 min. The GUS activity was localized histochemically incubating plant material in the staining buffer (100 mM sodium phosphate, pH 7.0; 1 mM EDTA, 0.05% Triton X-100, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$) containing 0.5 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in the dark at 37 °C for 8 h. After staining, tissues were fixed in a solution consisting of 45% ethanol, 5% formaldehyde, 5% acetic acid for 2 h at 4 °C and stored in 70% ethanol at 4 °C. GUS activities were inspected using a Stemi SV11 stereo microscope Axioscope (Zeiss, Jena, Germany) equipped with a video camera CCD3 (Sony, Japan).

Acknowledgements

We thank Prof. T. Nürnberger (University of Tübingen, Germany) for supply of systemin and systemin-17, Prof. W. Boland for supply of ethyl indanoyl conjugate, Prof. G. Howe (East Lansing, Michigan) for supply of *spr1* seeds, and Prof. C. Ryan (Pullman, Washington) for supply of OGAs. We express our thanks to Dr. B. McCaig (East Lansing, Michigan) for the English revision and C. Dietel (IPB Halle) for typing the manuscript. The technical assistance of K. Eichhorst and K. Klar (Biocenter) is gratefully acknowledged. N. Groß was supported by a Ph.D. grant of the Martin Luther University Halle-Wittenberg.

References

Abel, S., Köck, M., 2001. Secretory acid ribonucleases from tomato (*Lycopersicon esculentum* Mill.). In: Nicholson, A.W. (Ed.), Ribonucleases, Methods in Enzymology, vol. 341. Academic Press, New York, pp. 351–368.

Feussner, I., Wasternack, C., 2002. The lipoxygenase pathway. Annu. Rev. Plant Biol. 53, 275–297.

Green, T.R., Ryan, C.A., 1972. Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science 175, 776–777.

Hause, B., Stenzel, I., Miersch, O., Maucher, H., Kramell, R., Ziegler, J., Wasternack, C., 2000. Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. Plant J. 24, 113–126.

Hiraga, S., Ito, H., Sasaki, K., Yamakawa, H., Mitsuhara, I., Toshima, H., Matsui, H., Honma, M., Ohashi, Y., 2000. Wound-induced expression of a tobacco peroxidase is not enhanced by ethephon and suppressed by methyl jasmonate and coronatine. Plant Cell Physiol. 41, 165–170.

Howe, G.A., Lightner, J., Browse, J., Ryan, C.A., 1996. An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. Plant Cell 8, 2067–2077.

Howe, G.A., Lee, G.I., Itoh, A., Li, L., DeRocher, A.E., 2000. Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. Plant Physiol. 123, 711–724.

Jacinto, T., McGurl, B., Francheschi, V., Delano-Freier, J., Ryan, C.A., 1997. Tomato prosystemin promoter confers wound-inducible, vascular bundle-specific expression of the β-glucuronidase gene in transgenic tomato plants. Planta 203, 406–412.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W., 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6, 3901–3907.

Kessler, A., Baldwin, I.T., 2002. Plant responses to insect herbivory: the emerging molecular analysis. Annu. Rev. Plant Biol. 53, 299–328.

Köck, M., Groß, N., Stenzel, I., Hause, G., 2004. Phloem-specific expression of the wound-inducible ribonuclease LE from tomato (*Lycopersicon esculentum* cv. Lukullus). Planta, in press.

Köck, M., Löffler, A., Abel, S., Glund, K., 1995. Structural and regulatory properties of a family of starvation induced ribonucleases from tomato. Plant Mol. Biol. 27, 477–485.

Köck, M., Theierl, K., Stenzel, I., Glund, K., 1998. Extracellular administration of phosphate sequestering metabolites induces ribonucleases in cultured tomato cells. Planta 204, 404–407.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

LeBrasseur, N.D., MacIntosh, G.C., Perez-Amador, M.A., Saitoh, M., Green, P.J., 2002. Local and systemic wound-induction of RNase and nuclease activities in *Arabidopsis*: RNS1 as a marker for a JA-independent systemic signaling pathway. Plant J. 29, 393–403.

Lee, G.I., Howe, G.A., 2003. The tomato mutant *spr1* is defective in systemin perception and the production of a systemic wound signal for defense gene expression. Plant J. 33, 567–576.

Lehmann, K., Hause, B., Altmann, D., Köck, M., 2001. Tomato ribonuclease LX with the functional ER retention motif HDEF is expressed during physiological cell death processes including xylem differentiation, germination and senescence. Plant Physiol. 127, 436–449.

Leon, J., Rojo, E., Sanchez-Serrano, J.J., 2001. Wound signalling in plants. J. Exp. Bot. 52, 1–9.

Lers, A., Khalchitski, A., Lomaniec, E., Burd, S., Green, P.J., 1998. Senescence-induced RNases in tomato. Plant Mol. Biol. 36, 439–449.

Li, L., Li, C., Lee, G.I., Howe, G.A., 2002. Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato. Proc. Natl. Acad. Sci. USA 99, 6416–6421.

Li, C., Liu, G., Xu, C., Lee, G.I., Bauer, B., Ling, H.-Q., Ganal, M.W., Howe, G.A., 2003. The tomato *suppressor of prosystemin-mediated responses2* gene encodes a fatty acid desaturase required for the biosynthesis of jasmonic acid and the production of a systemic wound signal for defense gene expression. Plant Cell 15, 1646–1661.

Lowry, O.H., Rosebrough, A.L., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Malone, M., 1996. Rapid, long-distance signal transmission in higher plants. Adv. Bot. Res. 22, 163–228.

McGurl, B., Pearce, G., Orozco-Cardenas, M., Ryan, C.A., 1992. Structure, expression, and antisense inhibition of the systemin precursor gene. Science 255, 1570–1573.

Narváez-Vásquez, J., Ryan, C.A., 2004. The cellular localization of prosystemin: a functional role for phloem parenchyma in systemic wound signaling. Planta 218, 360–369.

O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O., Bowles, D.J., 1996. Ethylene as a signal mediating the wound response of tomato plants. Science 274, 1914–1917.

O'Donnell, P.J., Truesdale, M.R., Calvert, C.M., Dorans, A.M., Roberts, M.R., Bowles, D.J., 1998. A novel tomato gene that rapidly responds to wound-and pathogen-related signals. Plant J. 14, 137–142.

Orozco-Cardenas, M., Narváez-Vásquez, J., Ryan, C.A., 2001. Hydrogen peroxide acts as a second messenger for the induction

of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. Plant Cell 13, 179–191.

Pearce, G., Ryan, C.A., 2003. Systemic signaling in tomato plants for defense against herbivores. J. Biol. Chem. 32, 30044–30050.

Rhodes, J.D., Thain, J.F., Wildon, D.C., 1999. Evidence for physically distinct systemin signalling pathways in the wounded tomato plant. Ann. Bot. 84, 109–116.

Rojo, E., Titarenko, E., León, J., Berger, S., Vancanneyt, G., Sánchez-Serrano, J.J., 1998. Reversible protein phosphorylation regulates jasmonic acid dependent and independent wound signal transduction pathways in *Arabidopsis thaliana*. Plant J. 13, 153–165.

Ryan, C.A., 2000. The systemin signaling pathway: differential activation of plant defensive genes. Biochim. Biophys. Acta 1477, 112–121.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning. A Laboratory Mannual. Cold Spring Harbor Laboratory Press, New York.

Sasaki, K., Hiraga, S., Ito, H., Seo, S., Matsui, H., Ohashi, Y., 2002. A wound-inducible tobacco peroxidase gene expresses preferentially in the vascular system. Plant Cell Physiol. 43, 108–117.

Schüler, G., Görls, H., Boland, W., 2001. 6-Substituted indanoyl isoleucine conjugates mimic the biological activity of coronatine. Eur. J. Org. Chem. 546, 1663–1668.

Schüler, G., Mithöfer, A., Baldwin, I.A., Berger, S., Ebel, J., Santos, J.G., Herrmann, G., Hölscher, D., Kramell, R., Kutchan, T.M., Maucher, H., Schneider, B., Stenzel, I., Wasternack, C., Boland, W., 2004. Coronalon: a powerful tool in plant stress physiology. FEBS Lett. 563, 17–22.

Stankovic, B., Davies, E., 1996. Both action potential and variation potential induce proteinase inhibitor gene expression in tomato. FEBS Lett. 390, 275–279.

Stenzel, I., Hause, B., Maucher, M., Pitzschke, A., Miersch, O., Ziegler, J., Ryan, C.A., Wasternack, C., 2003. Allene oxide cyclase dependence of the wound response and vascular bundle specific generation of jasmonate in tomato-amplification in wound-signalling. Plant J. 33, 577–589.

Strassner, J., Schaller, F., Frick, U.B., Howe, G.A., Weiler, E.W., Amrhein, N., Macheroux, P., Schaller, A., 2002. Characterization and cDNA-microarray expression analysis of 12-oxophytodienoate reductases reveals differential roles for octadecanoid biosynthesis in the local versus the systemic wound response. Plant J. 32, 585–601.

Titarenko, E., Rojo, E., León, J., Sánchez-Serrano, J.J., 1997. JA-dependent and -independent signaling pathways control wound-induced gene activation in *Arabidopsis thaliana*. Plant Physiol. 115, 817–826.

Walling, L.L., 2000. The myriad plant responses to herbivores. J. Plant Growth Regul. 19, 195–216.

Wasternack, C., Hause, B., 2002. Jasmonates and octadecanoids: signals in plant stress responses and plant development. Progr. Nucleic Acid Res. Mol. Biol. 72, 165–221.

Wildon, K., Thain, J.F., Minchin, P.E.H., Gubb, I.R., Reilly, A.J., Skipper, Y.D., Doherty, H.M., O'Donnell, P.J., Bowles, D.J., 1992. Electrical signaling and systemic proteinase inhibitor induction in the wounded plant. Nature 360, 62–65.