Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 797–801
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Reduction of divinyl ether-containing polyunsaturated fatty acids in transgenic potato plants

Lennart Eschen-Lippold [a,1], Grit Rothe [a,1], Michael Stumpe [b], Cornelia Göbel [b], Ivo Feussner [b], Sabine Rosahl [a,*]

[a] *Department of Stress and Developmental Biology, Leibniz Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle/Saale, Germany*
[b] *Georg-August-University Göttingen, Albrecht-von-Haller Institute of Plant Sciences, Department of Plant Biochemistry, Justus-von-Liebig-Weg 11, D-37077 Göttingen, Germany*

Received 13 November 2006; received in revised form 11 December 2006
Available online 26 January 2007

Abstract

Oxygenated polyunsaturated fatty acids synthesized via the lipoxygenase pathway play a role in plant responses to pathogen attack. In solanaceous plants, the preferential stimulation of the 9-lipoxygenase pathway in response to pathogen infection leads to the formation of the divinyl ether-containing polyunsaturated fatty acids colneleic and colnelenic acid, as well as hydroxy and trihydroxy polyunsaturated fatty acids. To functionally assess the role of divinyl ethers, transgenic potato plants were generated which express an RNA interference construct directed against the pathogen-inducible 9-divinyl ether synthase. Efficient reduction of 9-divinyl ether synthase transcript accumulation correlated with reduced levels of colneleic and colnelenic acid. However, in response to infection with virulent *Phytophthora infestans*, the causal agent of late blight disease, no significant differences in pathogen biomass could be detected suggesting that the levels of antimicrobial divinyl ethers are not critical for defense against *Phytophthora infestans* in a compatible interaction.

Keywords: *Solanum tuberosum*; *Phytophthora infestans*; RNA interference; Oxylipin; Lipoxygenase pathway

1. Introduction

As signaling molecules and as antimicrobial secondary metabolites, oxylipins play a role in pathogen defense in plants (Rosahl and Feussner, 2004). Oxylipins arise as a consequence of autoxidation (Mueller, 2004) or are synthesized enzymatically *via* α-dioxygenases or the lipoxygenase (LOX) pathway (Feussner and Wasternack, 2002). Introduction of molecular oxygen into polyunsaturated fatty acids (PUFAs) such as linoleic (LA) and α-linolenic acid (LnA) by either 9- or 13-LOXs leads to the formation of 9-/13-hydroperoxy octadecadienoic acid (9-/13-HPOD) and 9-/13-hydroperoxy octadecatrienoic acid (9-/13-HPOT), respectively. The hydroperoxides are substrates for at least seven enzymes which catalyze the formation of epoxides, aldehydes, divinyl ethers or hydroxy PUFAs (Feussner and Wasternack, 2002).

Products of the LOX pathway have been implicated to play a role in pathogen defense as signaling molecules and as antimicrobial compounds. Jasmonic acid (JA), a product of the 13-LOX pathway, as well as its biosynthetic precursor 12-oxophytodienoic acid (OPDA) both act as

Abbreviations: CA, colneleic acid; CnA, colnelenic acid; CP-HPLC, chiral phase-HPLC; DES, divinyl ether synthase; GC, gas chromatography; HODE, hydroxy octadecadienoic acid; HOTE, hydroxy octadecatrienoic acid; HPODE, hydroperoxy octadecadienoic acid; HPOTE, hydroperoxy octadecatrienoic acid; JA, jasmonic acid; LOX, lipoxygenase; LA, linoleic acid; LnA, α-linolenic acid; PUFA, polyunsaturated fatty acid; OPDA, 12-oxophytodienoic acid; RP-HPLC, reversed phase-HPLC; SP-HPLC, straight phase-HPLC.

* Corresponding author. Tel.: +49 345 55821440; fax: +49 345 55811409.
E-mail address: sabine.rosahl@ipb-halle.de (S. Rosahl).

[1] These authors contributed equally to the work.

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.12.010

signaling molecules (Blechert et al., 1999; Kramell et al., 2000; Stintzi et al., 2001; Taki et al., 2005) and were shown to be essential for effective defense against a subset of pathogens and insects (Vijayan et al., 1998; McConn et al., 1997; Stintzi et al., 2001). An additional role for LOXs in membrane lipid peroxidation was postulated based on the observation that most of the hydroperoxides detected during the hypersensitive cell death were generated enzymatically by 9-LOXs (Rustérucci et al., 1999). However, in transgenic potato plants depleted of 9-LOX activity, enhanced autoxidation is observed and hypersensitive cell death appears unaltered (Göbel et al., 2003).

In potato, pathogen infection or elicitor treatment leads to the preferential stimulation of the 9-LOX pathway (Göbel et al., 2001, 2002). Apart from 9-hydroxy octadecadi(tri)enoic acid (9-HOD/T), products of the epoxy alcohol synthase pathway, 9,10,11- and 9,12,13-trihydroxy octadeca(di)enoic acid, and of the 9-divinyl ether synthase (9-DES) pathway (Fig. 1a), colneleic (CA) and colnelenic acid (CnA), accumulate in response to pathogen attack. Several lines of evidence suggest an important role of CA and CnA for the plant's response to pathogens. Firstly, antimicrobial activity of the 9-LOX-derived divinyl ethers has been demonstrated (Weber et al., 1999; Prost et al., 2005). This appears to be a specific effect since spore germination is inhibited to higher extents by treatment with CnA than CA (Prost et al., 2005). Secondly, accumulation of CA and CnA occurs earlier and to higher extents in potato cultivars with higher resistance against *Phytophthora infestans*, the causal agent of late blight disease of potato (Weber et al., 1999). Finally, application of CA reduces infection of barley by the powdery mildew *Blumeria graminis* f. sp. *hordei* (Cowley and Walters, 2005).

To address the role of CA and CnA in a functional manner, we modulated the levels of divinyl ether containing PUFAs in potato by expressing an RNA interference (RNAi) construct directed against the pathogen-inducible *9-DES* of potato. Interestingly, despite reduced levels of CA and CnA in the transgenic potato plants, growth of *P. infestans* was not affected. Thus, accumulation of divinyl ethers to high levels is apparently not required for basal resistance in potato.

2. Results and discussion

The divinyl ethers CA and CnA are synthesized from LA and LnA, respectively, by the action of a 9-LOX and a 9-DES (Itoh and Howe, 2001; Stumpe et al., 2001; Fig. 1a). In order to specifically reduce the levels of CA and CnA in potato plants, an RNAi construct was generated using a 348 bp fragment of the coding region of potato *9-DES* (Stumpe et al., 2001). The fragment was cloned in antisense orientation upstream, and in sense orientation downstream, of a truncated GUS gene behind the 35S pro-

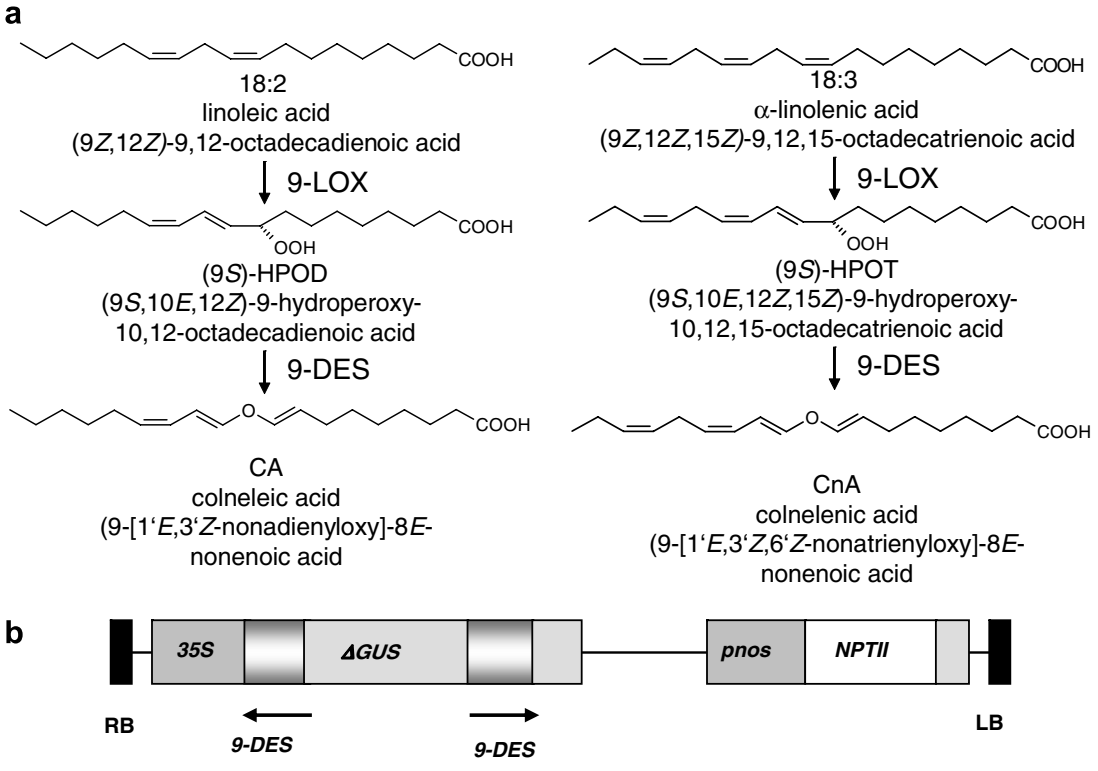

Fig. 1. (a) Schematic presentation of the synthesis of CA and CnA in plants. (b) Structure of the *9-DES*-RNAi construct. 35S: 35S promoter, *GUS*: coding region of the ß-glucuronidase, LB: left border of the T-DNA, *NPTII*: neomycin phosphotransferase, pnos: nopaline synthase promoter, RB: right border of the T-DNA.

moter in a binary vector (Fig. 1b). Potato plants (*Solanum tuberosum* cv. Désirée) were transformed with *Agrobacterium tumefaciens* containing the binary vector with the 35S-*9-DES*-RNAi construct. Transgenic plants were regenerated and checked by Southern analyses for the presence of the transgene (Fig. 2a and b). In order to analyze whether *9-DES* expression was successfully reduced in the transgenic plants, leaves were infiltrated with *Pseudomonas syringae* pv. *maculicola*. This treatment leads to high expression of *9-DES* after 6–12 h (Stumpe et al., 2001). In two independent lines of the resulting transgenic plants (V1 and V2), *9-DES* transcript levels were significantly lower compared to those detected in wild type plants 6 and 12 h after bacterial infiltration (Fig. 2c), indicating that the endogenous *9-DES* mRNA was effectively degraded by RNAi.

We have previously shown that the products of the 9-DES reaction, CA and CnA, start to accumulate 6–12 h after infiltration of *P. syringae* pv. *maculicola*, reaching maximal levels after 24 and 36 h (Göbel et al., 2002). Therefore, levels of CA and CnA were determined in extracts from both transgenic and wild-type plants 24 h after infiltration with *P. syringae* pv. *maculicola* (Fig. 3). Concomitant with the decrease in *9-DES* transcript levels, the amount of the divinyl ether containing PUFA CnA was significantly reduced in the two transgenic lines V1 and V2 compared to the wild-type plants.

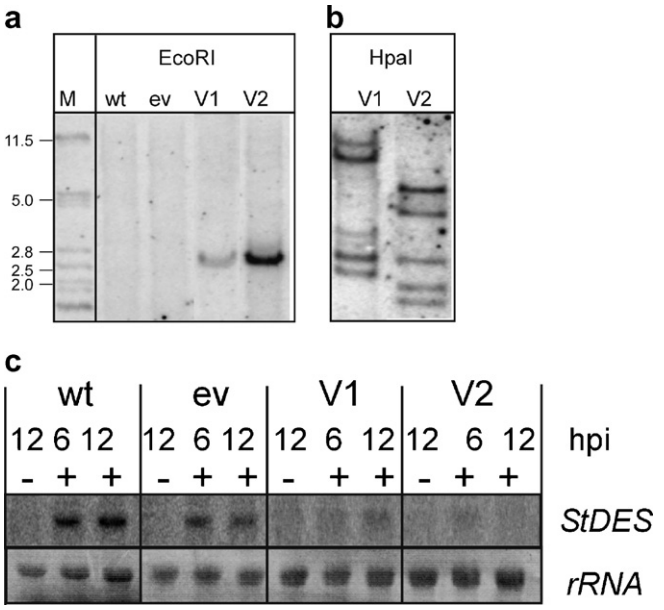

Fig. 2. Characterization of transgenic *9-DES*-RNAi plants. (a, b): Southern analysis with genomic DNA from plants V1 and V2. DNA was digested with *Eco*RI (a) or *Hpa*I (b), separated by agarose gel electrophoresis and subjected to Southern analysis. As radioactively labeled probes, *uidA* (a) and the RNAi fragment (b) were used. Panel a shows the integrated construct as a single band of the expected size. Panel b demonstrates that V1 and V2 are independent lines. (c) Down-regulation of *9-DES* expression in transgenic *9-DES*-RNAi plants after pathogen infection. Leaves of wild type (wt) and transgenic plants carrying an empty vector cassette (ev) or the *9-DES*-RNAi construct (V1 and V2) were infiltrated with 10 mM $MgCl_2$ (−) or *P. syringae* pv. *maculicola* (+). RNA was isolated at the time points indicated and subjected to Northern analysis. Hybridization was carried out with radioactively labeled cDNAs for *StDES* or *rRNA* as indicated.

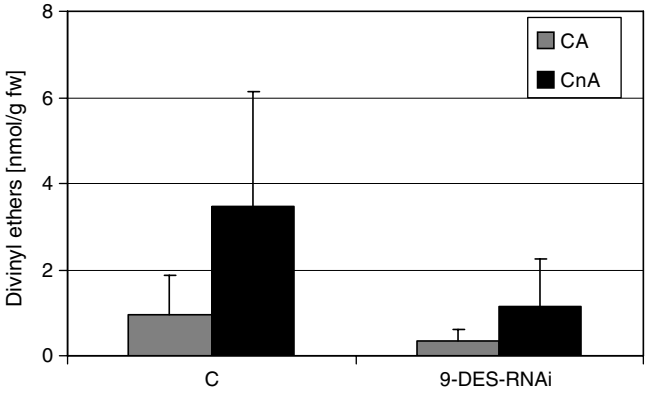

Fig. 3. Reduced levels of CA and CnA in transgenic plants expressing a *9-DES*-RNAi construct. The levels of CA (gray bars) and CnA (black bars) were analyzed in potato leaves 24 h after infiltration with *P. syringae* pv. *maculicola*. Data shown are combined from wild type and transgenic plants carrying an empty vector cassette ("C") or the *9-DES*-RNAi construct (V1 and V2, "9-DES-RNAi") and were obtained in four independent experiments (paired *t*-test: $p = 0.058$, $n = 8$ for CA; $p = 0.03$, $n = 8$ for CnA).

The efficient reduction of 9-LOX-derived compounds was also observed in transgenic potato plants expressing an RNAi construct targeted at the pathogen-induced *9-LOX* of potato (Göbel et al., 2003). In these *9-LOX*-RNAi plants, reduced levels of 9-HPOD/T and 9-HOD/T as well as reduced levels of CA and CnA were obtained. Interestingly, despite drastically reduced 9-LOX activity, lipid peroxidation still occurred suggesting that, in the absence of 9-LOX activity, autoxidative processes are the predominant cause of lipid peroxidation (Göbel et al., 2003).

The importance of 9-LOXs for successful pathogen defense in solanaceous plants was demonstrated in tobacco. Here, antisense suppression of the pathogen-induced *9-LOX* led to a suppression of race-cultivar-specific resistance against *Phytophthora parasitica* var. *nicotianae* (Rancé et al., 1998). However, the mechanism of how 9-LOXs are involved in conferring resistance is not known. We therefore tested transgenic plants depleted in the 9-LOX-derived divinyl ether containing PUFAs, CA and CnA, for alterations in their susceptibility to *P. infestans*. Infection of the susceptible potato cultivar Désirée with *P. infestans* results in a compatible interaction which is characterized by the rapid development of lesions on the leaves and significant mycelial growth of the oomycete within 3 days. Infection assays were carried out with both control plants and the *9-DES*-RNAi plants V1 and V2. In addition to the assessment of the disease symptoms (Fig. 4a), a quantitative real time PCR assay was used to determine the biomass of the pathogen (Fig. 4b). Primers were derived from a repetitive DNA element of *P. infestans* (Judelson and Tooley, 2000) and used in a Taqman-based PCR reaction. In three independent experiments, no statistically significant alterations in

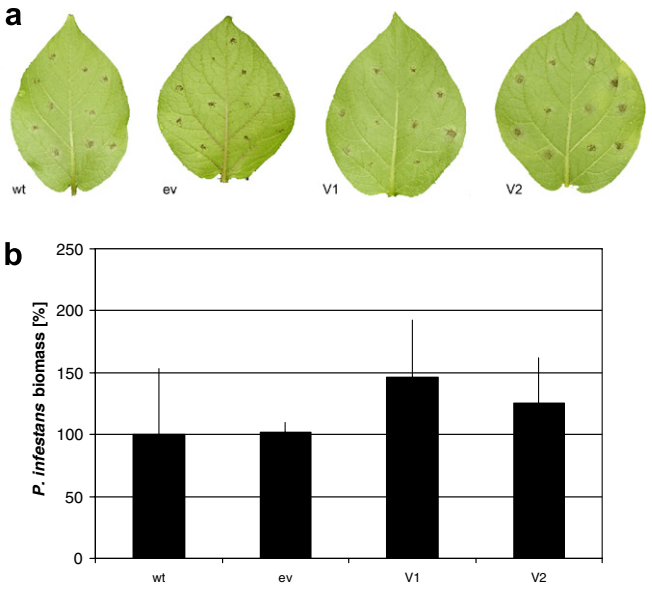

Fig. 4. Growth of *P. infestans* on transgenic plants. Leaves of wild type (wt) and transgenic plants carrying the empty vector (EV) or the *9-DES*-RNAi construct (V1 and V2) were infected with 1×10^5 *P. infestans* zoospores/ml. Pathogen biomass was determined 3 days after infection by Real Time PCR with DNA isolated from infected leaves using *P. infestans*-specific primers.

pathogen biomass could be determined (Fig. 4b), suggesting that the reduction in CA and CnA levels does not affect susceptibility towards *P. infestans*. These results suggest that the products of the 9-LOX pathway, CA and CnA, are either not required at all, possibly due to their late accumulation during infection (Göbel et al., 2002), or that the low residual amounts of divinyl ethers are still sufficient for basal defense responses. Since, on the other hand, 9-LOXs are of importance for race-cultivar-specific resistance in tobacco (Rancé et al., 1998), analyses of resistant potato plants expressing *9-DES*-RNAi constructs will show whether depletion of divinyl ethers negatively affects successful defense responses.

3. Experimental

3.1. Cloning of an StDES-RNAi construct

The *StDES*-RNAi construct was cloned in an analogous manner to the 9-LOX-RNAi construct (Göbel et al., 2003). Briefly, a 348 bp fragment was amplified from the *StDES* cDNA using the primers 5′-AAATCTTCCGATTCGTG-3′ and 5′-GCTGCATGGTTTGGATC-3′. The RNAi construct comprising the StDES fragments cloned in front of and behind a truncated GUS gene were inserted into a binary vector carrying the 35STX promoter (Gatz et al., 1992) and the resulting plasmid was transferred to *Agrobacterium tumefaciens* GV3101. Potato plants were transformed with recombinant agrobacteria and transgenic plants were regenerated as described (Feltkamp et al., 1995).

3.2. Infection of plants and analysis of defense responses

Sterile potato plants (*Solanum tuberosum* L. cv. Désirée) were grown in tissue culture in a phytochamber with 16 h of light [140 μE] at 22 °C. Plants were transferred to soil and grown in a phytochamber with 16 h of light [140 μE], 20 °C and 60% humidity for 4 weeks. Suspensions of *P. syringae* pv. maculicola at a concentration of 10^8 cfu/ml in 10 mM $MgCl_2$ or, as a control, 10 mM $MgCl_2$-solution were infiltrated into lower leaves. For *P. infestans* infections, lower leaves were drop-inoculated with a *P. infestans* zoospore-suspension in water (1×10^5 zoospores/ml) on the abaxial leaf surface and kept at 100% humidity for the duration of the experiment. As controls, water was pipetted onto the leaves.

3.3. Determination of P. infestans biomass

Quantitative real time PCR was applied to determine the growth of *P. infestans* on wild type and transgenic potato plants. DNA was isolated from six leaf disks of 6 mm diameter each using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), after addition of external standard DNA. The primers PIO8-3-3F (5′-CAATTCGCCACCTTCTTCGA-3′) und PIO8-3-3R (5′-GCCTTCCTGCCCTCAAGAAC-3′; MWG Biotech, Ebersberg, Germany) as well as the Taqman®-MGB-NFQ probe PIO8-3-3M1: 6-FAM-5′-CGTACGGCCAATGTA-3′-MGB-NFQ (Applied Biosystems, Darmstadt) were used to amplify and detect a *P. infestans*-specific repetitive DNA sequence (Judelson and Tooley, 2000) with the ABI Prism Sequence Detection System (Applied Biosystems, Darmstadt, Germany). For standard curves, a larger DNA fragment was amplified from *P. infestans* genomic DNA with the primers O8-3 (5′-TAACC-GACCAAGTAGTAAA-3′) and O8-4 (5′-GAAAGGCAT AGAAGGTAGA-3′; Judelson and Tooley, 2000) and cloned into the vector pCR2.1 (Invitrogen, Karsruhe, Germany). PCR was carried out using the Taqman® Universal PCR Master Mix (Applied Biosystems, Roche, New Jersey, USA) with 10′ at 95 °C followed by 40 cycles with 15″ at 95 °C and 1′ at 60 °C each. Relative amounts of *P. infestans* DNA in the samples were calculated using the ABI Prism 7000 Sequence Detection-Software version 1.1 by including the external standard and the data from the standard curve. Statistical analyses were performed using One-Way Anova.

3.4. Determination of products of the LOX pathway

Determination of divinyl ethers was performed as described (Göbel et al., 2003).

Acknowledgements

The excellent technical assistance of Angelika Weinel is gratefully acknowledged. This work was supported by the EU (QLK5-CT-2001-02445 to I.F. and S.R.) and by the DFG (DFG 446/1 to I.F. and SFB 648 TPA4 to S.R.).

References

Blechert, S., Bockelmann, C., Fusslein, M., von Schrader, T., Stelmach, B., Niesel, U., Weiler, E.W., 1999. Structure-activity analyses reveal the existence of two separate groups of active octadecanoids in elicitation of the tendril-coiling response of *Bryonia dioica* Jacq. Planta 207, 470–479.

Cowley, T., Walters, D., 2005. Local and systemic effects of oxylipins on powdery mildew infection in barley. Pest. Manag. Sci. 61, 572–576.

Feltkamp, D., Baumann, E., Schmalenbach, W., Masterson, R., Rosahl, S., 1995. Expression of the mannopine synthase promoter in roots is dependent on the *mas* elements and correlates with high transcript levels of *mas*-binding factor. Plant Sci. 109, 57–65.

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

Gatz, C., Frohberg, C., Wendenburg, R., 1992. Stringent repression and homogeneous de-repression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants. Plant J. 2, 397–404.

Göbel, C., Feussner, I., Schmidt, A., Scheel, D., Sanchez-Serrano, J., Hamberg, M., Rosahl, S., 2001. Oxylipin profiling reveals the preferential stimulation of the 9-lipoxygenase pathway in elicitor-treated potato cells. J. Biol. Chem. 276, 6267–6273.

Göbel, C., Feussner, I., Hamberg, M., Rosahl, S., 2002. Oxylipin profiling in pathogen-infected potato leaves. Biochem. Biophys. Acta 1584, 55–64.

Göbel, C., Feussner, I., Rosahl, S., 2003. Lipid peroxidation during the hypersensitive response in potato in the absence of 9-lipoxygenases. J. Biol. Chem. 278, 52834–52840.

Itoh, A., Howe, G.A., 2001. Molecular cloning of a divinyl ether synthase – Identification as a CYP74 cytochrome P-450. J. Biol. Chem. 276, 3620–3627.

Judelson, H.S., Tooley, P.W., 2000. Enhanced polymerase chain reaction methods for detecting and quantifying *Phytophthora infestans* in plants. Phytopathology 90, 1112–1119.

Kramell, R., Miersch, O., Atzorn, R., Parthier, B., Wasternack, C., 2000. Octadecanoid-derived alteration of gene expression and the "oxylipin signature" in stressed barley leaves. Implications for different signaling pathways. Plant Physiol. 123, 177–188.

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E., Browse, J., 1997. Jasmonate is essential for insect defense in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 94, 5473–5477.

Mueller, M.J., 2004. Archetype signals in plants: the phytoprostanes. Curr. Opin. Plant Biol. 7, 441–448.

Prost, I., Dhondt, S., Rothe, G., Vicente, J., Rodriguez, M.J., Kift, N., Carbonne, F., Griffiths, G., Esquerre-Tugaye, M.T., Rosahl, S., Castresana, C., Hamberg, M., Fournier, J., 2005. Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. Plant Physiol. 139, 1902–1913.

Rancé, I., Fournier, J., Esquerré-Tugayé, M.-T., 1998. The incompatible interaction between *Phytophthora parasitica* var. *nicotianae* race 0 and tobacco is suppressed in transgenic plants expressing antisense lipoxygenase sequences. Proc. Natl. Acad. Sci. USA 95, 6554–6559.

Rosahl, S., Feussner, I., 2004. Oxylipins. In: Murphy, D.J. (Ed.), Plant Lipids: Biology, Utilisation and Manipulation. Blackwell Publisher, Oxford, pp. 329–354.

Rustérucci, C., Montillet, J.L., Agnel, J.P., Battesti, C., Alonso, B., Knoll, A., Bessoule, J.J., Etienne, P., Suty, L., Blein, J.P., Triantaphylidès, C., 1999. Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves. J. Biol. Chem. 274, 36446–36455.

Stintzi, A., Weber, H., Reymond, P., Browse, J., Farmer, E.E., 2001. Plant defense in the absence of jasmonic acid: The role of cyclopentenones. Proc. Natl. Acad. Sci. USA 98, 12837–12842.

Stumpe, M., Kandzia, R., Göbel, C., Rosahl, S., Feussner, I., 2001. A pathogen-inducible divinyl ether synthase (CYP74D) from elicitor-treated potato suspension cells. FEBS Lett. 507, 371–376.

Taki, N., Sasaki-Sekimoto, Y., Obayashi, T., Kikuta, A., Kobayashi, K., Ainai, T., Yagi, K., Sakurai, N., Suzuki, H., Masuda, T., Takamiya, K., Shibata, D., Kobayashi, Y., Ohta, H., 2005. 12-Oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in Arabidopsis. Plant Physiol. 139, 1268–1283.

Vijayan, P., Shockey, J., Levesque, C.A., Cook, R.J., Browse, J., 1998. A role for jasmonate in pathogen defense of *Arabidopsis*. Proc. Natl. Acad. Sci. USA 95, 7209–7214.

Weber, H., Chetelat, A., Caldelari, D., Farmer, E.E., 1999. Divinyl ether fatty acid synthesis in late blight-diseased potato leaves. Plant Cell 11, 485–493.