Free Radical Research, June 2005; 39(6): 637–647

Relationship among lipoperoxides, jasmonates and indole-3-acetic acid formation in potato tuber after wounding

MASSIMO REVERBERI[1], CORRADO FANELLI[1], SLAVEN ZJALIC[1], STEFANIA BRIGANTI[2], MAURO PICARDO[2], ALESSANDRA RICELLI[3], & ANNA ADELE FABBRI[1]

[1]*Dipartimento Biologia Vegetale, Università "La Sapienza", Largo Cristina di Svezia, 24, Rome 00165, Italy,* [2]*Istituto Dermatologico San Gallicano, IRCCS, via San Gallicano 25, Rome 00153, Italy, and* [3]*Institute of Sciences of Food Production, CNR, Via Amendola 122, Bari 70125, Italy*

Accepted by Dr. J. Yodoi

(Received 19 June 2004; in revised form 19 January 2005)

Abstract

Plant responses to biotic and abiotic stress can be mediated by oxidised products and in this study we analysed the relation among some of them and the growth factor indole-3-acetic acid (IAA). The plant material used was potato tuber sliced below bud and incubated for different lengths of time before analysis. Wounding in potato tuber leads, in a very short time (0–30 min), to the generation of lipid hydroperoxides (LOOH) from polyunsaturated fatty acids (PUFA). These reactive species could cause a subsequent increase of 9 and 13-lipoxygenase (LOX, E.C.1.13.12.12.), analysed by RT-PCR and spectrophotometric assay, LOOH, Jasmonates and IAA all quantified by GC–MS analysis. The activation of 9 and 13-LOX, using different timing, leads to the formation of LOOH with a subsequent generation of jasmonates and IAA as highlighted by the addition on the potato tuber slices of salicylhydroxamic acid (SHAM), an inhibitor of LOX activity. A correlation between jasmonates and IAA resulted by testing their reciprocal influence during wounding in potato tuber. The relationship occurring among each hormone analysed during wounding underlines the fact that the jasmonates level can be regulated *in situ* and this can suggest a role for these compounds in potato tuber which has been underestimated up to now.

Keywords: *Potato tuber, wounding, hormones, stress-related compounds, lipid hydroperoxides*

Abbreviations: AA, arachidonic acid; BHT, butylated hydroxytoluene; C18:2, linoleic acid; C18:3, linolenic acid; C18:2OOH, peroxide of linoleic acid; 9-HODE, 9-hydroxy-10(E),12(Z)-octadecadienoic acid; 13-HODE, 13-hydroxy-9(Z),11(E)-octadecadienoic acid; IAA, indole-3-acetic acid; JA, jasmonic acid; LOOH, lipoperoxides; LOX, lipoxygenase; MJ, methyl jasmonate; PL, polar lipids; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid.

Introduction

In plants hydroperoxy derivatives of polyunsaturated fatty acids (PUFA) and their oxidized products (oxylipins), like eicosanoids in animals, are involved in the signalling pathway related to development as well as biotic and abiotic stress responses [1–5]. Both in animals and in plants a key role in the enzymatic peroxidation of PUFA is played by lipoxygenases (LOX) and cycloxygenase [4], even if oxidized compounds can also be formed non-enzymatically [6–9]. All the plants present different isoforms of LOX enzymes with different specific activity in relation to pH, pI and substrates, in particular, in potato, 12 9-*lox* genes are present and one of them seems to be specifically involved in tuber formation

Correspondence: M. Reverberi, Dipartimento Biologia Vegetale, Università "La Sapienza", Largo Cristina di Svezia, 24, 00165, Rome, Italy. Tel: 39 0 649917125/6. Fax: 39 0 66833878. E-mail: massimo.reverberi@uniroma1.it

ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/10715760500072115

[10]. Furthermore the different cellular localization suggests that multiple physiological functions are carried out by this enzyme [11]. In potato plant their expression is tissue- or organ-specific and it is modulated during its development, in response to abiotic stress (i.e. wounding), to pathogen infection or jasmonic acid (JA) and methyl jasmonate (MJ) treatments [12]. The LOX products can be toxic at high concentrations, whereas lower amounts could act as secondary messengers in plant growth and development [13]. Turner et al. (2002), [14] suggest a complex network between the LOX pathway and stress compounds such as JA, MJ, traumatin, systemin, abscissic acid and ethylene. Jasmonates, formed by LOX action as defense responses, are involved both in biotic and abiotic stresses and in some aspects of plant development [15]. Moreover a relationship between jasmonates, abscissic acid, ethylene and indole-3-acetic acid (IAA) has been suggested [14,16]. Studies on *A. thaliana* mutants (*coi1, tir1, jar1, axr1-24*) showing an altered response to IAA and JA application, demonstrated a link between JA and IAA signalling pathway [14]. On the contrary in potato tuber several studies have been carried out on LOX enzymes and their products but the relationship between them and IAA has not been fully investigated.

Lipoperoxides (LOOH) derived from LOX action can stimulate morphogenesis in potato tuber, either directly (JA and MJ formation), or indirectly through the modulation of IAA production, implying a close relationship between LOOH and IAA [2,17]. In potato tuber slices (cv Kennebec) arachidonic acid (AA) induced browning and phytoalexins accumulation during hypersensitive response, associated with cell death, nuclear hypertrophy, tracheary element differentiation and cell wall lignification [2,18,19]. We have shown that peroxides of linoleic and linolenic acid which originates from LOX activity were involved in early events (0–2 h) occurring in potato tubers after wounding as mitosis, periderm formation, starch hydrolysis and bud initiation mediated by IAA formation [2]. In addition, during cold storage (4°C) of potato tuber (cv Kennebec) up to 6 months, LOOH and IAA accumulation are responsible for budding [3]. In this study we analyse the ability of the potato tuber after harvesting to carry on jasmonates biosynthesis and we attempt to correlate jasmonates *neo*-synthesis with IAA formation considering the relationship between LOOH and IAA [2], LOOH and JA.

Materials and methods

Plant material

Certified potato tubers (*Solanum tuberosum* L. cv Kennebec, supplied by PAT-FRUT, Castel D'Aiano, Bologna, Italy) were stored at 4°C and were brought to room temperature 24 h prior to use. The wounding procedure was carried out as previously described [3].

Lipid analysis

Potato tuber slices, 5 g fresh weight (f.w.) each, were washed four times in sterilized distilled water, then extracted three times with 10 ml of chloroform:methanol (2:1 v/v) in the presence of 100 μg of butylated hydroxytoluene (BHT) as an antioxidant, as previously reported [2]. The extracted lipids were fractionated by thin layer chromatography and the fraction of the polar lipid (PL) was recovered as previously reported [20]. Tricosanoic acid (C23:0) was used as internal standard. The recovered PL fractions were trans-esterified by boron-trifluoride (BF_3 10% in CH_3OH, Carlo Erba). The analysis of fatty acid methyl-esters was carried out by gas chromatography (GC) on a capillary column FFA-P 50 m × 0.32 mm × 0.52 μm as reported [21] to detect the percentage of C18:2 and C18:3 in the sample.

IAA, jasmonic acid and methyl jasmonate detection

The detection of IAA, JA and MJ were performed at different times (0, 10, 20, 30, 60 min, 6 and 24 h after wounding) extracting 5 g f.w. of tuber sliced below-bud. The extraction was carried out three times for 1 h with 10 ml ethyl acetate and BHT 30 μM as an antioxidant. A measure of 5 μg of 2,4 dichlorophenoxyacetic acid (2,4 D, Sigma) and 5 μg of dihydro-JA (dh-JA), obtained from methyl dh-JA (Bedoukian res.) after hydrolysis, were used as internal standards. After the extraction, the samples were filtrated (Whatmann filter paper no 4) and the filtrates were evaporated under N_2 stream and then silylated. Standard solutions of MJ were carried out by derivatization of JA with diazomethane and the obtained MJ was quantified by GC–MS as reported below. In other experiments IAA, JA and MJ concentration was monitored under the same experimental conditions after the addition to the potato slice of 20 μl of IAA 10 μM or MJ 10 μM or JA 10 μM or the LOX inhibitor salicylhydroxamic acid (SHAM, 1 mM) solutions. The trimethyl-silyl-ether (TMS)-derivatives were analysed as previously reported [3]. Quantitative analyses were performed in single ion monitoring (SIM) mode, selecting the ions having *m/z* 319, 304, 202 and 292, 257, 219 for the trimethylsilyl derivative of IAA and 2,4-D respectively, and ions of *m/z* 282, 223; 224, 193; 284, 225 for the derivatives of JA, MJ and dh-JA, respectively. Calibration curves were constructed by plotting the ratios of the integrated peak areas of IAA and 2,4 D, JA, MJ and dh-JA against their amount and performing a linear regression using equal weighting. The method showed a good linearity

($R > 0.99$) in the analysed concentration range of 1–50 ng/μl for each compound tested.

Lipid hydroperoxide detection

LOOH formation was detected at different times (0, 10, 20, 30, 60 and 120 min and 6, 24 h) after wounding on lipids extracted, as reported in the previous section, from 5 g f.w. of tuber slices. The presence of total LOOH was assayed spectrophotometrically using N,N-diethyl-1,4-phenylenediammoniumsulphate (DEPD) according to Nazzaro-Porro et al. [22].

Lipoxygenase spectrophotometric assay

LOX activity was measured, following the formation of the conjugated dienes by the increase in absorbance at 234 nm using a Beckman DU530 spectrophotometer, as reported [2]. Potato tuber slices (5 g) were homogenized in 0.1 M acetate buffer (pH 4.5), NaCl 500 mM, dithiothreitol 2 mM, ethylendiaminetetraacetic disodium salt 1 mM, glycerin 10% w/v, ascorbic acid 5 mM, phenylmethylsulfonyl fluoride 1 mM. The homogenate was centrifuged at 12,000*g* for 15 min at 4°C and the supernatant is collected. The quantity of proteins in the supernatants were determined by Bradford reagent (Sigma). LOX activities were measured in a final volume of 2 ml containing Tris–HCl 0.1 M (pH 7.0) in untreated or IAA-treated samples, Tris–HCl 0.1 M (pH 6.0) or acetate buffer 0.1 M (pH 5.0), 100 μl supernatant, 180 μM C18:2 or C18:3 as substrates. In order to exclude the possible interference of peroxidase activity, KCN 1 mM was added to the reaction mixture before the spectrophotometric assay.

Analysis of lipoxygenase products in potato tubers

The LOX reaction with C18:2 was performed as previously described. The products generated were reduced with $NaBH_4$ 15 mM for 5 h at room temperature. After reduction, the samples were acidified to pH 3.0 with HCl 1 M and extracted three times with ethyl-acetate, dried under a nitrogen flow and 9- and 13-hydroxylinoleic acid were separated by HPLC as reported [23], recovered and then methylated with diazomethane and silylated overnight with TMS at room temperature. The products derived were analysed separately using a GC–MS method and quantified using 9-HODE and 13-HODE as standard (Cayman Chemical) using a Restek 5 capillary column. The oven temperature was programmed from 150°C to 250°C at a rate of 10°C/min and held for 20 min with an injector temperature of 220°C. The analyses were performed in SIM mode, selecting the ions having *m*/*z* 382, 225 and 311 for both 9-HODE and 13-HODE.

Cloning and sequencing of the S. tuberosum tuber aoc and lox alleles

DNA extracted from the tuber of *S. tuberosum* cv Kennebec was amplified in a thermal Eppendorf Mastercycler gradient following these amplification steps (94°C × 2 min; [94°C × 30 s, 56°C × 45 s, 72°C × 1 min] × 35 times; 72°C × 8 min) using primers LOX1-1 (forward 5′ ATTGGTCAAATTACGAGCGG 3′; reverse 5′ GTAGGCTGGATTGCTGTGCT 3′), LOX1-2 (forward 5′ GTGGGTGGACTAATTGGTGG 3′; reverse 5′ GCCAAGGGCCTCAAAGAT 3′), LOX1-3 (forward 5′ CATCCGAATGGACAAAGGAT 3′; reverse 5′ AAATGAAACTTGACGATGTGGA 3′), designed on the non conserved nucleotides of 5′-UTR region of the three alleles of *lox1* [24], StLOX H3 (forward 5′ AAAAACCCAGAAGAAGCACC 3′; reverse 5′ ATTTGGATCCATGTCAGTGC 3′) designed on the consensus conserved nucleotides region of the *loxH3* [11] and StAoc (forward 5′ ATCTYGTYCCMTTYASCAACAA 3′; reverse 5′ TCRCCRAAGTAGGAAGCTGTA 3′) designed on the consensus conserved region of *aoc* sequences deriving from several solanaceae found in GenBank. The amplification in high stringency conditions gave a unique band for the four *lox* and for *aoc* (100, 120, 180, 420 and 450 bp, respectively). These fragments were cloned in pGEMT easy vector (Promega) sequenced and aligned with TBLASTX 2.1 software of the NCBI website (www.ncbi.nlm.nih.gov/BLAST). The result of the Blast indicate a high homology of *Stlox1-1, 2, 3* with LOX-1 types, of *StloxH3* with LOX-3 type [11] and of *Staoc* with allene oxide cyclase from *Solanum tuberosum* and other solanaceae. New specific primers StkLox1-1 (*Solanum tuberosum* cv kennebec) (forward 5′ TACGGGCGGACTTATTTTTG 3′: reverse 5′ GAAAAGAAACCTTTTGGCCC 3′), StkLox1-2 (forward 5′ GTGGGTGGACTAATTGGTGG 3′; reverse 5′ TCAGTCAAAGAACCGCAAGA 3′), StkLox1-3 (forward 5′ AACCCATTGCTGCTTTTGAG 3′; reverse 5′ TCCTTTGCCTGTGAGTCCTT 3′), StkLoxH3 (forward 5′ AAAAACCCAGGCGAAGCACT 3′; reverse 5′ ATTTGGATGCATGTCAGAGT 3′) Stkaoc (forward 5′ ATTTTCCTGCAAGAGCCAGA 3′; reverse 5′ TTCGTGCTTGATCAGAATGC 3′) were designed and used for the subsequent RT-PCR analysis. The *Stklox1-1, 1-2, 1-3* fragments generated ranged from 100 to 200 bp subsequently the couple of 18S primers used as internal standard St18S1 (forward 5′ TAGATAAAAGGTCGACGCGG 3′; reverse 5′ CCCAAAGTCCAACTACGAGC 3′) originated a single fragment of 400 bp, while the primer chosen for the amplification of *StkloxH3* and *Staoc* originated two single fragment of 420 and 400 bp, respectively. For this RT-PCR analysis, another couple of 18S primers were used

(St18S2; forward 5′ ATGATAACTCGACGGATCGC; reverse 5′ CTTGGATGTGGTAGCCGTTT 3′) that gave a unique band at 200 bp [25].

9-lox, 13-lox and aoc specific semi-quantitative RT-PCR analysis

Total RNA from 100 mg of freeze-dried potato tuber sliced below the bud was extracted using Tri- Reagent protocol (Sigma) at different times after wounding (0, 10, 20, 30, 60 min and 6, 24 h) and was quantified spectrophotometrically by determining the optical density at 260 nm. RNA was treated with RNAse-free Dnase I kit (Sigma) resuspended in 20 μl of DEPC-treated water and the extracts were used to develop a *lox* and *aoc* RT-PCR assay. RT was performed using 500 ng of total RNA, 200U of Superscript reverse transcriptase (Invitrogen) and following the manufacturer's instructions. The RT reaction mixture (0.5 μl) was used for *lox*-specific and *aoc*-specific PCR amplification together with 10 pmol of each of the *S. tuberosum lox*- and *aoc*-specific primers. The program included 30 cycles consisting of 95°C for 30 s, 58°C for 20 s and 72°C for 1 min. RT-PCR control reaction mixtures contained either water or 10 ng of *Solanum tuberosum* cv Kennebec genomic DNA. Constitutive expression of ribosomal 18S RNA was tested by using RNA extracts from *S. tuberosum* in all the time points. A semi-quantitative [25] *lox* RT-PCR was used to study *S. tuberosum lox's* and *aoc* expression in all the time points. In other experiments the expression of *Staoc* and *StkloxH3* were monitored under the same experimental conditions after the addition to the potato slice of IAA (10 μM) and this was compared with the control. The ratio of *lox* and *Staoc* to 18S PCR products was determined using Molecular analyst software (Bio-Rad) and this ratio was used as an index of the relative *lox* and *Staoc* mRNA expression in samples.

Northern blot analysis of StloxH3 and Staoc

Total RNA extraction, from 100 mg of freeze-dried potato tuber sliced below the bud, was carried out, at different times after wounding (0, 10, 20, 30, 60 min and 6, 24 h) on untreated and IAA treated (10 μM) samples as described in the section of the specific semi-quantitative RT-PCR analysis. An aliquot (5 μl) of total RNA was visualised on 1.5% agarose gel after ethidium bromide staining and the quality (260/280 nm ratio) and quantity of RNA (OD 260 nm) was measured by Perkin–Elmer DU5780 spectrophotometer. An equal quantity of total RNA (10 μg) was used for the subsequent mRNA purification, performed by the GenElute mRNA miniprep kit (Sigma-Aldrich). mRNA from each time point and treatment was transferred to nylon membranes and hybridised with probe for gene encoding *StloxH3* and *Staoc*. The probes to detect *StloxH3* and *Staoc* (420 and 450 bp, respectively) mRNAs were derived from cDNA amplification with specific primers using the PCR DIG labelling system (Roche). mRNA was separated by 4 h electrophoresis in 1.5% MOPS gel at 30 V and transferred to a N^+ nylon membrane (Positive charged nylon membrane, Roche Diagnostics GmbH, Mannheim, Germany). Hybridisation was carried out at 55°C according to Roche DIG hybridisation manual.

Statistical analysis

In all experiments, mean values were compared using Student's *t*-test.

Results

Decrease of PUFA in polar lipids and LOOH production in potato tuber slices at different times after wounding

Linoleic and linolenic acid are the main PUFA present in potato tuber and both can be substrates for LOX activity. In previous work [2] the trend of PUFA and LOOH was monitored for 120 min detecting an inverse relationship between them. In these novel experiments a significant ($P < 0.001$) decrease of C18:2 + C18:3 percentage in the PL was observed at 20 min (30%) and at 6 h (50%) after wounding (Figure 1) associated with an LOOH formation. A good inverse correlation ($R = -0.92$; $P < 0.001$) between PUFA percentage and LOOH amount occurred until 120 min. After this time point LOOH increased and at 24 h reached a value similar to the value reported at 20 min, while PUFA, after a further decrease at 6 h, maintained the same value at 24 h. This value was lower compared to time 0 (Figure 1).

Time course of LOX activities at different times after wounding

The spectrophotometric analysis of LOX enzymes was carried out at pH 5.0, 6.0 and 7.0 using 180 μM linoleic (C18:2) or α-linolenic acid (C18:3) as substrates. In the presence of C18:2 (Figure 2a), LOX activity peaked at 20 min ($52.0 \pm 4.2\ U\ mg^{-1}$) at pH 5.0 and 6.0, while a slighter LOX activity is reported at pH 7.0. The analysis carried out at pH 7.0, starting from 30 min, has shown a higher activity in comparison to that measured at pH 5.0 and 6.0 (Figure 2a). LOX activities evaluated with C18:3 (Figure 2b) were lower at the same pHs, in comparison to C18:2 and among these, the LOX(s) working at pH 7.0 is the most relevant. When IAA was added to potato tuber slices, an increase of LOX activity on C18:3 at 30 min and 6 h was detected, in comparison with the untreated control (Table I).

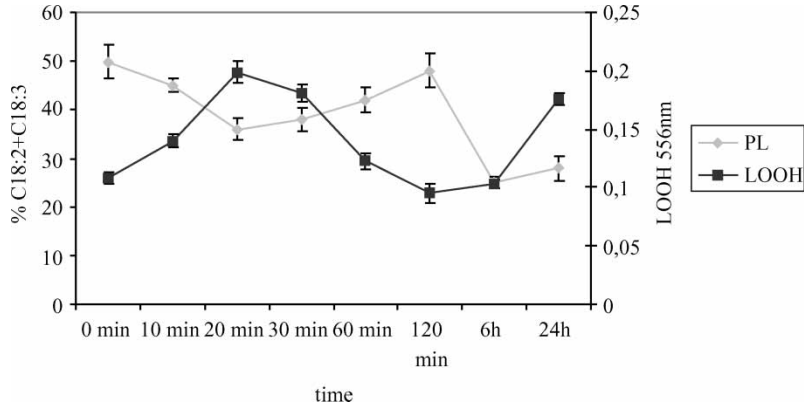

Figure 1. Percentage of C18:2 + C18:3 of PL fraction and LOOH formation (Abs 556 nm) in potato tuber slices at different times (0, 10, 20, 30, 60 and 120 min, 6 and 24 h) after wounding. The data represent the mean ± S.E.M. from $n = 5$ potato tuber slices for each time point from three separate experiments.

Analysis of 9 and 13-HODE produced by LOX action at pH 7.0 at different times after wounding

A 9-LOX activity is prevalent at pH 7.0 during the time after wounding as shown in Table II, in fact the percentage of 9-HODE was always higher than 13-HODE each time it was tested. The percentage of 9-HODE was similar at all the time points even if a little increase was shown at 30 min and 24 h in comparison with time 0. The quantity of 9 and 13-HODE produced by LOX action, increased after 30 min (575.5 ± 12.5 and 206.1 ± 10.2, respectively) and 6 h (505.5 ± 18.2 and 211.8 ± 11.3, respectively).

9-lox and 13-lox specific semi-quantitative RT-PCR analysis

In the semi-quantitative RT-PCR analysis in *S. tuberosum* cv Kennebec, inclusion of primer 18S forward and reverse in *Stklox1-3* RT reaction mixtures

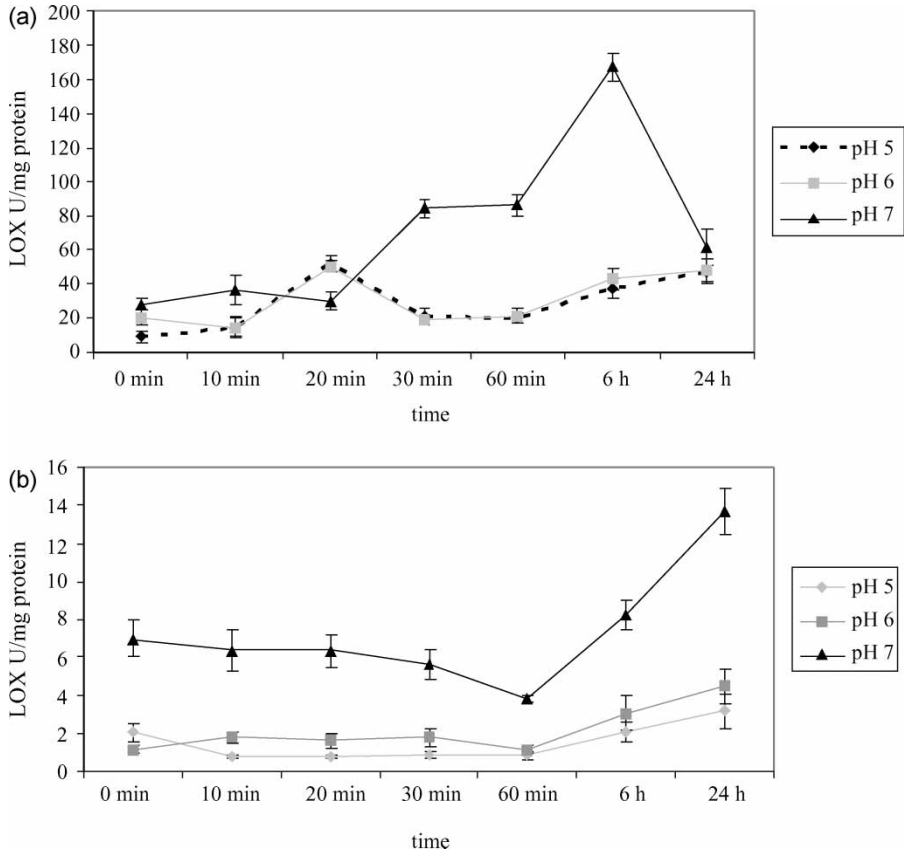

Figure 2. LOX activities in potato tuber detected at pH 5.0, 6.0 and 7.0 using 180 μM C18:2 (a) or C18:3 (b) as substrates at different times (0–24 h) after wounding. The data represent the mean ± S.E.M. from $n = 3$ potato tuber slices for each time point from three separate experiments.

Table I. Analysis of LOX activity (U/mg protein) using C18:3 as substrate, at different times (0,10, 20, 30 and 60 min, 6 and 24 h) on wounded potato tuber untreated or treated with IAA (10 μM).

	CONT	IAA
0 min	5.9 ± 0.5	6.6 ± 0.5
10 min	6.0 ± 0.9	5.6 ± 0.5
20 min	5.7 ± 0.6	3.3 ± 0.3
30 min	6.2 ± 0.3	20.7 ± 0.7
60 min	9.7 ± 0.7	7.3 ± 0.8
6 h	12.8 ± 0.8	17.0 ± 1.0
24 h	15.0 ± 0.9	9.5 ± 1.2

The data represent the mean ± S.E.M. from $n = 3$ potato tuber slices for each time point from three different experiments.

resulted in the generation of two bands, the 180 bp *Stklox1-3* fragment and the 420 bp-fragment of 18S ribosomal RNA (Figure 3a), while in the RT-PCR analysis of *StkloxH3* RT reaction mixtures resulted in the generation of two bands, the 450 bp *StkloxH3* fragment and the 250 bp-fragment of 18S ribosomal RNA. The results obtained with RT-PCR assay showed that the *StkLox1-3*, a 9-LOX, was activated at all the time points, while *StkLoxH3*, a 13-LOX, was activated early (0–20 min) and late (6 h) times after wounding. Moreover in potato slices treated with IAA, an increase in *StkLoxH3* transcription at 30 min and 6 h is evident (Figure 3 a,b). These results support the data obtained with enzymatic activity allowing us to hypothesize that both 9 and 13-LOX are involved in wounding in potato tubers. *Stklox1-1* and *1-2* presented a very slight expression that did not seem connected with wounding events (data not shown).

Northern blot analysis of 13-lox

The data obtained with RT-PCR analysis on *13-lox* were further verified using Northern blot method. The results obtained (Figure 3a) confirmed the stimulation of *StkloxH3* mRNA (3.0 Kb) expression exerted by IAA observed at 30 min and 6 h in the RT-PCR analysis, supporting the role played by this enzyme in the formation of precursors of jasmonates.

Table II. Percentage and quantity of 9 and 13-HODE after the action of LOX, extracted from potato slices at different times after wounding (0, 10, 20, 30 and 60 min, 6 and 24 h), on C18:2 (180 μM) at pH 7.0.

	9-HODE		13-HODE	
Time	(%)	(ng/g f.w.)	(%)	(ng/g f.w.)
0	71.4 ± 2.3	391.5 ± 5.6	28.6 ± 1.2	184.3 ± 4.2
10	74.2 ± 2.1	322.1 ± 6.3	25.9 ± 1.5	131.7 ± 3.2
20	75.8 ± 3.2	372.0 ± 8.5	24.2 ± 2.2	139.5 ± 6.2
30	76.6 ± 3.5	575.5 ± 12.5	23.4 ± 2.3	206.1 ± 10.2
60	73.5 ± 4.1	432.5 ± 11.2	26.5 ± 1.5	183.0 ± 5.6
6 h	73.7 ± 2.3	505.5 ± 18.2	26.3 ± 1.2	211.8 ± 11.3
24 h	77.5 ± 3.3	258.8 ± 10.3	22.5 ± 1.3	88.0 ± 2.3

The data represent the mean ± S.E.M. from $n = 3$ potato tuber slices for each time point from three different experiments.

JA, MJ and IAA formation in potato tuber slices at different times after wounding

The time course of IAA, JA and MJ at different times after wounding (0, 10, 20, 30 and 60 min, 6 and 24 h) is reported in Figure 4. All the compounds increased early (20–30 min) and late (6–24 h) time points with a different trend: In the early times, IAA peaked at 20 min before JA and MJ (30 min) and increased significantly at 24 h, even if in other experiments IAA peaked at 6 h (Figure 5a). JA and MJ always peaked at 6 h.

Analysis of JA, MJ and IAA in presence of LOX inhibitor SHAM

SHAM, an inhibitor of LOX activity, decreased LOOH formation [2], thus by its use the influence of LOOH on jasmonates and IAA production can be studied. In Table III the effect of SHAM (1 mM) added to potato slice on jasmonates and IAA production was reported. At 6 h after wounding JA (151.2 ± 9.8 and 52.9 ± 4.1, control and treated samples, respectively), MJ (control 13.8 ± 0.4 and SHAM 5.0 ± 0.3) and IAA (15.5 ± 2.1, control; 7.0 ± 0.7, SHAM) formation was inhibited by the presence of SHAM and this effect was maintained up to 24 h on all the compounds analysed.

Relationship between jasmonates and IAA

Some experiments have been carried out to investigate if there is a relationship between jasmonates and IAA could be evidenced. When potato tuber slices were treated with MJ (Figure 5a), IAA formation was stimulated at early (10 min, about 10 fold) and late time points (6 h, about 1.4 fold), while the addition of JA enhanced IAA production only at 6 h (about 2 fold). The influence of IAA on MJ production was more evident starting from 6 to 24 h (Figure 5b). IAA led to a stimulation of JA up to 30 min, followed a trend similar to the control from 60 min to 24 h (Figure 5c).

Northern blot and RT-PCR analysis of Staoc mRNA levels in the control and after IAA addition on potato tuber slices

In concordance ($R = 0.97$; $P < 0.001$) with the early stimulation of JA on IAA-treated potato slices (Figure 5c), the addition of IAA on potato, highly enhances *Staoc* mRNA transcription at early times (10–30 min) (Figure 6 a,b). In the control a very low expression of this gene can be detected. There are no significant differences between control and IAA-treated samples from 60 min to 24 h. To further

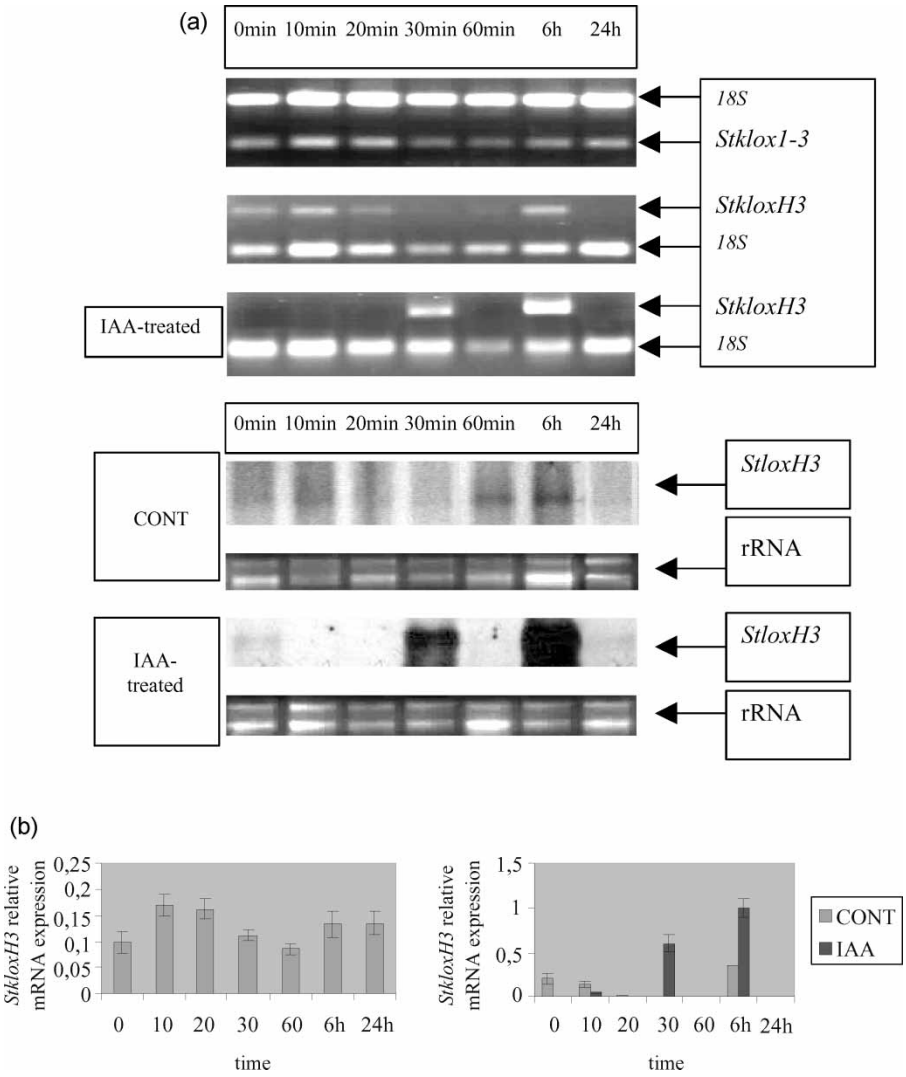

Figure 3. RT-PCR analysis of *Stklox1-3 (9-lox)*. RT-PCR and Northern blot analysis of *StkloxH3 (13-lox)* mRNA levels of transcription were analysed from potato tuber slices, untreated or IAA-treated (10 μM), at different times after wounding (0–24 h) (a). In the graphs below (b), the mean ± S.E.M. of the relative mRNA expression of the different *lox* are reported. Each assay represents RNA from $n = 5$ potato slices from three separate experiments.

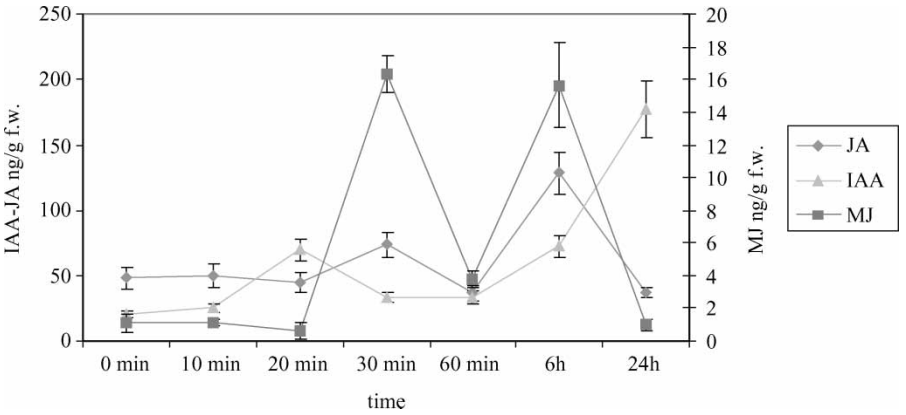

Figure 4. Analysis of jasmonates (JA and MJ) and IAA at different times after wounding (0, 10, 20, 30, 60 min, 6, 24 h). The jasmonates and IAA were extracted from $n = 5$ potato slices for each time point. The data represent the mean ± S.E.M. of three different experiments.

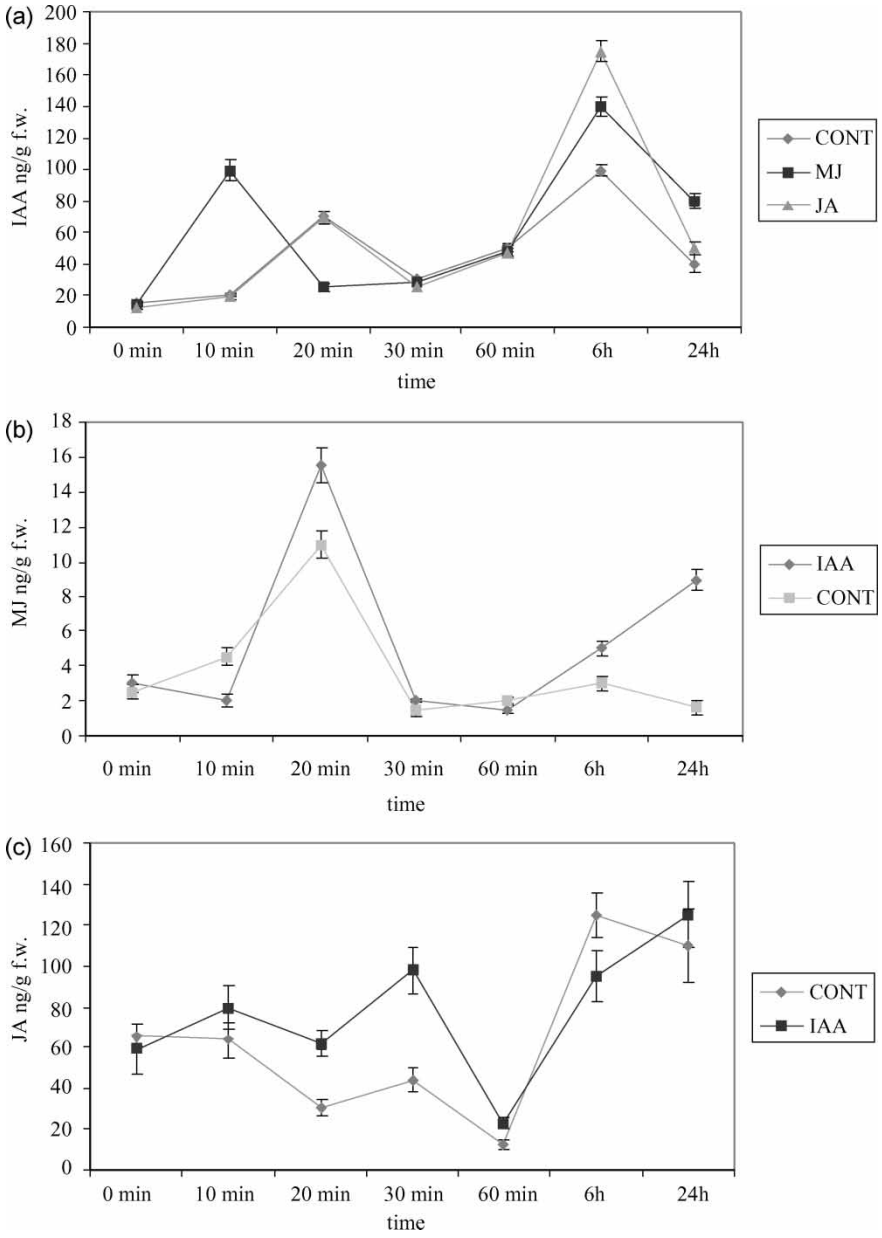

Figure 5. Relationship among MJ, JA and IAA in potato after wounding. In (a) is shown the effect of MJ (10 μM) and JA (10 μM) addition on IAA production, in (b) the effect of IAA (10 μM) addition on MJ production and in (c) the effect of IAA (10 μM) treatment on JA production. The data were evaluated on $n = 5$ potato slices for each time point and represent the mean ± S.E.M. of three different experiments.

confirm the data obtained by RT-PCR analysis a northern blot analysis on mRNA was performed. As was shown in Figure 5a there is a substantial overlap between the two molecular approaches. In the case of *Staoc* mRNA (~0.8 Kb) expression (Figure 5a), this was hardly detectable in the control through a northern blot analysis confirming the very slight activation showed also through the RT-PCR analysis.

Table III. Effect of the LOX inhibitor SHAM (1 mM) on jasmonates and IAA formation at 6 and 24 h.

	IAA (ng/g f.w.)		JA (ng/g f.w.)		MJ (ng/g f.w.)	
Time	CONT	SHAM	CONT	SHAM	CONT	SHAM
6 h	15.5 ± 2.1	7.0 ± 0.7	151.2 ± 9.8	52.9 ± 4.1	13.8 ± 0.4	5.0 ± 0.3
24 h	179.5 ± 9.4	44.0 ± 2.2	71.5 ± 6.2	54.6 ± 3.8	6.5 ± 0.4	3.7 ± 0.2

The data represent the mean ± S.E.M of three experiments.

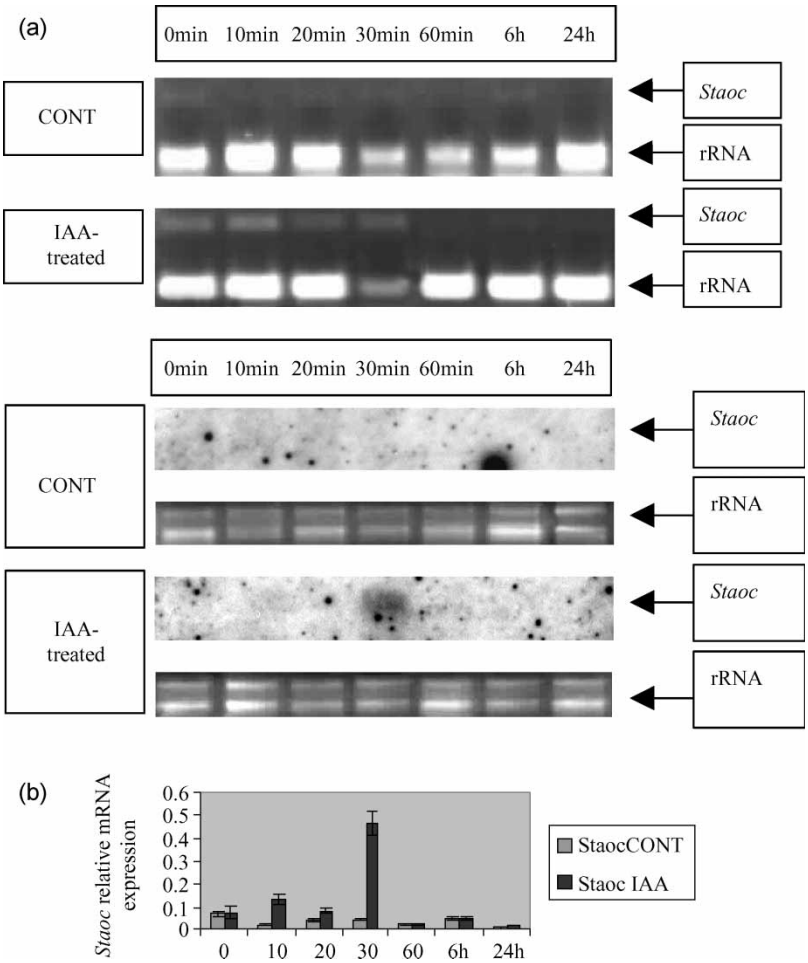

Figure 6. RT-PCR and Northern blot analysis of *Staoc* evaluated in untreated and IAA-treated (10 μM) potato tuber slices at different times (0, 10, 20, 30, 60 min, 6, 24 h) after wounding (a). The relative *Staoc* mRNA expression in the time course was reported in (b). The data represents the mean ± S.E.M. from five potato slices for each time point of three separate experiments.

The stimulation occurring by IAA treatment on *Staoc* activation was further confirmed by the hybridisation, as shown in Figure 5a, in fact, an increase of *Staoc* mRNA levels at 30 min in the IAA-treated samples in comparison with the control can be evidenced.

Discussion

The LOX and oxylipins metabolism in plants is a very branched *route* which has been extensively reviewed over the last few of years [26–27]. Nevertheless, as some authors have pointed out [14], the interrelationship among the different components and their modulation in the net is still unclear.

This work attempts to investigate some aspects of the relationship between LOX, LOOH, jasmonates and IAA in a particular environmental condition such as the abiotic stress (wounding) in potato tubers. Lipoxygenase can modulate the production of LOOH by acting on different PUFA (C18:2 and C18:3) and originating products with different regio-specificity (9 and 13 LOOH) [27–28]. In wounded potato tuber different LOX enzymes work at different pH (5.0–7.0) levels, but a prevalent 9-LOX activity is evident at all pH levels and each time it was tested, confirming the results obtained by other authors [26]. The accumulation of 9-LOOH may have antimicrobial action [11,26] and could improve the defence response of potato tuber tissue after wounding. However, we can not rule out other roles of these reactive species as Kolomiets et al. (2001) pointed out, ascribing to 9-LOOH a role in the development of the potato tuber [10]. We also found a *13-lox* expressed during the time course, which could account for 13-S-hydroperoxylinolenic acid (13S-HPOTE) formation from C18:3, the precursor of JA, by 13-LOX action. The formation of this stress-hormone has been extensively studied in potatoes and its biosynthesis is supposed to occur in green leaves and from this bulk, distributed in the other parts of the plant. The JA necessary for tuber formation seems to originate from this bulk [29]. In our study a formation of JA is evident between 0 and 24 h:

The JA level at time 0 probably derives from the bulk originated in the leaves but, after wounding, an induced *de novo* synthesis seems to occur suggesting that this compound can also play a role in the tuber after the harvest. In a previous study [2], which we carried out in the same experimental conditions, we reported an increase at 6 h of mitosis in tuber periderm (about 29 fold) and in the bud dome (5 fold). These morphogenic events could be ascribed also to JA increase as in other plants [15,30]. In fact *13-lox (Stklox-H3)* and *aoc* expression increased at 6 h when the highest formation of JA is highlighted, moreover LOX activity on C18:3 resulted higher at this point in comparison with early times. JA could be responsible for the activation of *lox* transcription [14,17] and the little bulk of JA produced up to 30 min could induce a LOX leading to the accumulation of precursors necessary for the late (6 h) JA peak. Using LOX inhibitor SHAM, a significant ($P < 0.001$) inhibition of JA formation at 6 h after wounding occurs, supporting the role played by 13-LOX in the late events as suggested by the increase of LOX activity on linolenic acid (Figure 2b) and 13-HODE formation (Table II) at the same time point. Moreover SHAM addition inhibits IAA formation at early [2] and at late times, confirming that a relation exists between lipoperoxidation and auxin formation in these experimental conditions. The IAA increase at 6 h could be related to jasmonates production, as suggested by the stimulation of IAA in jasmonates-treated tuber slices (Figure 5a). Otherwise MJ addition could induce IAA-Ala hydrolase, a MJ-responsive gene [17], responsible for the release of IAA from its conjugated form. IAA addition affects JA formation at early times (0–30 min) as confirmed by the enhancement of *13-lox* and allene oxide cyclase expression (*aoc*), key steps in JA biosynthesis. A recent hypothesis indicates the presence of a IAA response element in the promoters of JA-related genes [31].

By our results the defence responses to wounding during the time in potato tuber tissues could be modulated through an interrelationship between jasmonates and IAA. In our opinion the correlation between these compounds leads to an alteration of the concentration of each molecule that in turn regulates the formation of the other. Supporting this hypothesis, *A. thaliana* mutants (*axr1, axr1-3, axr1-24, jar1, coi1*), defective in the IAA-signal transduction are insensitive to jasmonates. Furthermore some JA-responsive genes (*lox2, aos* and *Atvsp*) are induced by IAA in *axr1* [14,32]. The defence reaction of the potato tuber seems to be related to a cascade of events starting with the generation of reactive species which modulate an enzymatic system which produces signal compounds inducing physiological responses [9]. These early signals could trigger the switching on of an enzymatic system in which LOX activation, LOOH production, JA, MJ and IAA formation are involved.

References

[1] Vijayan P, Shockey J, Levesque CA, Cook RJ, Browse J. A role for jasmonate in pathogen defense of *Arabidopsis*. Proc Natl Acad Sci USA 1998;95:7209–7214.

[2] Fabbri AA, Fanelli C, Reverberi M, Ricelli A, Camera E, Urbanelli S, Rossini A, Picardo M, Altamura MM. Early physiological and cytological events in potato tuber after wounding. J Exp Bot 2000;51:1267–1275.

[3] Reverberi M, Picardo M, Ricelli A, Camera E, Fanelli C, Fabbri AA. Oxidative stress, growth factor production and budding in potato tubers during cold storage. Free Radic Res 2001;35:833–841.

[4] Ziboh VA, Cho Y, Mani I, Xi S. Biological significance of essential fatty acids/prostanoids/lipoxygenase-derived monohydroxy fatty acids in the skin. Arch Pharm Res 2002;25(6):747–758.

[5] Lim H, Dey SK. A novel pathway of prostacyclin signaling-hanging out with nuclear receptors. Endocrinology 2002;143(9):3207–3210.

[6] Siedow JN. Plant lipoxygenase: Structure and function. Annu Rev Plant Phys 1991;42:145–188.

[7] Croft KPC, Voisey CR, Slusarenko AJ. Mechanism of hypersensitive cell collapse: Correlation of increased lipoxygenase activity with membrane damage in leaves of *Phaseolus vulgaris* (L.) inoculated with an avirulent race of *Pseudomonas syringae* pv *phaseolicola*. Physiol Mol Plant Pathol 1990;36:49–62.

[8] Berger S, Weichert H, Porzel A, Wasternack C, Kuhn H, Feussner I. Enzymatic and non-enzymatic lipid peroxidation in leaf development. BBA 2001;1533:266–276.

[9] Spiteller G. Peroxidation of linoleic acid and its relation to aging and age dependent diseases. Mech Ageing Dev 2001;122:617–657.

[10] Kolomiets MV, Hannapel DJ, Chen H, Tymeson M, Gladon RJ. Lipoxygenase is involved in the control of potato tuber development. Plant Cell 2001;13:613–626.

[11] Royo J, Vancanneyt G, Perez AG, Sanz C, StÖrmann K, Rosahl S, Sanchez-Serrano JJ. Characterization of three potato lipoxygenases with distinct enzymatic activities and different organ-specific and wound-regulated expression patterns. J Biol Chem 1996;271(35):21012–21019.

[12] Porta H, Rocha-Sosa M. Plant lipoxygenase physiological and molecular features. Plant Physiol 2002;130:15–21.

[13] Jones AM. Surprising signalling in plant cells. Science 1994;263:183–184.

[14] Turner JG, Ellis C, Devoto A. The jasmonate signal pathway. Plant Cell 2002;14:S153–S164.

[15] Creelman RA, Mullet JE. Jasmonic acid distribution and action in plants: Regulation during development and response to biotic and abiotic stress. Proc Natl Acad Sci USA 1995;92:4114–4119.

[16] Leon J, Rojo E, Sanchez-Serrano JJ. Wound signalling in plants. J Exp Bot 2001;52(354):1–9.

[17] Sasaki Y, Asamizu E, Shibata D, Nakamura Y, Kaneko T, Awai K, Amagai M, Kuwata C, Tsugane T, Masuda T, Shimada H, Takamiya K, Ohta H, Tabata S. Monitoring of methyl-jasmonate-responsive genes in *Arabidopsis* by cDNA macroarray: Self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signalling pathway. DNA Res 2001;8:153–161.

[18] Altamura MM, Castoria R, Fabbri AA, Tomassi M, Fanelli C. Cytological events induced by arachidonic acid in potato tubers. New Phytol 1994;128:57–62.

[19] Castoria R, Fanelli C, Fabbri AA, Passi S. Metabolism of arachidonic acid involved in its eliciting activity in potato tuber. Physiol Mol Plant Pathol 1992;41:127–137.

[20] Passi S, Nazzaro-Porro M, Picardo M, Finotti E, Fabbri AA, Fanelli C. Microsomal and mitochondrial involvement in

production of aflatoxin induced by carbon tetrachloride and hydroperoxide in cultures of *Aspergillus parasiticus*. Trans Brit Mycol Soc 1986;87(3):451–456.

[21] Passi S, De Luca C, Fabbri AA, Brasini S, Fanelli C. Possible role of ergosterol oxidation in aflatoxin production by *Aspergillus parasiticus*. Mycol Res 1994;98(3):363–368.

[22] Nazzaro-Porro M, Passi S, Picardo M, Mercantini R, Breathnach AS. Lipoxygenase activity of *Pityrosporum in vitro* and *in vivo*. J Investig Dermatol 1986;87(1):108–112.

[23] Mita G, Gallo A, Greco V, Zasiura C, Casey R, Zacheo G, Santino A. Molecular cloning and biochemical characterization of a lipoxygenase in almond (*Prunus dulcis*) seed. Eur J Biochem 2001;268:1500–1507.

[24] Kolomiets MV, Chen H, Gladon RJ, Braun EJ, Hannapel DJ. A leaf lipoxygenase of potato induced specifically by pathogen infection. Plant Physiol 2000;124:1121–1130.

[25] Doohan FM, Weston G, Rezanoor HN, Parry DW, Nicholson P. Development and use of a reverse transcription-PCR assay to study expression of *Tri5* by *Fusarium* species *in vitro* and in planta. Appl Environ Microbiol 1999;65: 3850–3854.

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

[27] Feussner I, Wasternack C. The lipoxygenase pathway. Annu Rev Plant Biol 2002;53:275–297.

[28] Brash AL. Lipoxygenases: Occurrence, functions, catalysis, and acquisition of substrate. J Biol Chem 1999;274(34): 23679–23682.

[29] Koda Y. Possible involvement of jasmonates in various morphogenic events. Physiol Plantarum 1997;100:639–646.

[30] Biondi S, Scaramagli S, Capitani F, Altamura MM, Torrigiani P. Methyl jasmonates upregulates biosynthetic gene expression, oxidation and conjugation polyamines, and inhibits shoot formation in tobacco thin layers. J Exp Bot 2001;52:231–242.

[31] Devoto A, Turner JG. Regulation of jasmonate-mediated plant response in *Arabidopsis*. Ann Bot 2003;92:329–337.

[32] Lincoln C, Britton JH, Estelle M. Growth and development of the *axr*1 mutants of *Arabidopsis*. Plant Cell 1990;2:1071–1080.