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Metabolic profiling of oxylipins upon salicylate treatment in barley leaves — preferential induction of the reductase pathway by salicylate[1]

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Abstract In barley leaves, 13-lipoxygenases (13-LOXs) are induced by salicylate (SA) and jasmonate. Here, we show by metabolic profiling that upon SA treatment, free linolenic acid and linoleic acid accumulate in a 10:1 ratio reflecting their relative occurrence in leaf tissues. Furthermore, 13-LOX-derived products are formed and specifically directed into the reductase branch of the LOX pathway leading mainly to the accumulation of (13*S*,9*Z*,11*E*,15*Z*)-13-hydroxy-9,11,15-octadecatrienoic acid (13-HOT). Under these conditions, no accumulation of other products of the LOX pathway has been found. Moreover, exogenously applied 13-HOT led to *PR1b* expression suggesting for the time a role of hydroxy polyenoic fatty acid derivatives in plant defense reactions.

Key words: Hydroxy polyenoic fatty acid; Lipoxygenase; Hydroperoxide reductase; Volatile aldehyde; *Hordeum vulgare*

1. Introduction

Adaptation of higher plants to biotic and abiotic stress is often accompanied by the occurrence of lipid peroxidation and metabolites which derived therefrom. They are collectively called oxylipins [1]. Lipid peroxidation may be the result of a controlled action of enzymes or the result of autoxidation. The initial step of enzymatic lipid peroxidation is the dioxygenation of polyunsaturated fatty acids (PUFAs) by lipoxygenases (LOXs) either at carbon atom C-9 (9-LOX) or at carbon atom C-13 (13-LOX) of linolenic acid (LeA) or linoleic acid (LA), respectively [2]. These LOX-derived hydroperoxy PUFAs (HPOD or HPOT) can be converted in different reactions of the LOX pathway [1] (Fig. 1): (i) by a peroxygenase (POX) or reductase leading to hydroxy PUFAs (HOD or HOT), (ii) by a LOX leading to keto PUFAs, (iii) by a divinyl ether synthase (DES) leading to vinyl ether-containing PUFAs, (iv) by an allene oxide synthase (AOS) leading to jasmonic acid (JA) and (v) by a hydroperoxide lyase (HPL) leading to ω-keto fatty acids and aldehydes by fragmentation of the fatty acid molecule. Most of these LOX-derived compounds are regarded to function in plant defense reactions suggested by the following facts: (i) defense-related genes are induced by C_6 volatiles [3], (ii) HPL-derived aldehydes and POX-derived hydroxy PUFAs exhibit antimicrobial activity [4,5], (iii) divinyl ethers such as colnelenic acid accumulate upon pathogen infection and exhibit antimicrobial activity [6], (iv) JA is an important signalling compound and is involved in some responses to pathogens as shown by JA-insensitive mutants which are more susceptible [7,8], and (iv) transgenic plants with down-regulated LOX-mRNA by antisense expression were more susceptible to pathogens [9,10].

Salicylate (SA) was shown to play a critical role in the activation of defense gene expression leading to the establishment of the systemic acquired resistance (SAR) [11,12]. In many plants, a pathogen attack leads to an endogenous rise of SA followed by expression of pathogenesis-related (PR) genes such as *PR1*. Beside a SA-dependent pathway, induced systemic resistance (ISR) can be formed without endogenous rise of SA [13]. There are accumulating evidences on the existence of different defense signalling pathways in which SA, JA or ethylene are independent signals but having cross-talk to each other [7,14,15]. However, the puzzle is given by the facts that in tomato leaves, SA was found to inhibit JA biosynthesis upstream of 12-oxo-phytodienoic acid (OPDA) [16] and to block JA-responsive gene expression downstream of JA [17]. Furthermore, SA increases AOS mRNA accumulation in *Arabidopsis* leaves [18] and flax leaves [19], and the AOC activity from maize kernels is not inhibited by SA in vitro [20]. Due to the OPDA accumulation upon SA treatment of *Arabidopsis* leaves, release of OPDA from chloroplasts, where OPDA is formed, was suggested to be inhibited by SA [18].

Furthermore, SA may regulate the key reaction of oxylipin formation, which is catalyzed by one or several LOXs, as well as the ratio of the different branches of the LOX pathway (Fig. 1). Indeed, in barley leaves, at least one specific LOX is transcriptionally activated by SA and JA [21]. This LOX of 100 kDa (LOX-100) is a 13-LOX [22] and is located in the chloroplast [23]. SA treatment also induces *PR1b* expression in barley leaves (K.-H. Kogel, personal communication), and the co-induction of LOX-100 may suggest a role in plant defense reactions. However, LOX-100 is not expressed upon infection with powdery mildew in susceptible and non-susceptible barley lines [21]. In barley leaves, SA does not lead to AOS mRNA accumulation (H. Maucher and C. Wasternack, unpublished data) and JA formation [21]. However, the SA-induced *LOX* expression should elevate levels of (13*S*,9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT), the primary 13-*LOX* product derived from the conversion of LeA (Fig. 1). Since there is no SA-induced JA formation after 24 h of SA treatment [21], we analyzed the formation of metabolites of other branches of the LOX pathway upon SA treatment. By recording profiles of representative metabolites of the different branches, we have found a

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preferential induction of the reductase pathway upon SA treatment of barley leaves, indicating that metabolites derived from enzymatic lipid peroxidation may be a part of the response to SA.

2. Materials and methods

In all experiments, primary leaves of 7-day-old seedlings of barley (*Hordeum vulgare* L. cv. Salome) were used. Growth of seedlings, extraction of proteins and RNA and Northern blot analysis were performed as described [21,24]. Treatments with SA, JAME or 13-HOT were performed by floating leaf segments of 5 cm length on 50 μM solution at 25°C under continuous white light (120 μmol photon/m^2/s) provided by a fluorescent lamp (NARVA) for indicated times. Immunoblot analysis was performed as described [22].

13-HOT was prepared by reduction of 13-HPOT with sodium borohydride in 60% (by volume) methanol and purified by straight phase high performance liquid chromatography (HPLC) (SP-HPLC) as described below. 13-HPOT was prepared as described before [25].

The analysis of LOX-derived products from barley leaves was performed as described before using some modifications [26]. 1 g of frozen leaf tissue was added to 20 ml of extraction medium (hexane/isopropanol, 3/2, by volume, with 0.0025%, by weight, butylated hydroxytoluene) and was immediately homogenized with an Ultra Turrax for 30 s under a stream of argon on ice. The extract was centrifuged at 4500×*g* at 4°C for 15 min. The clear upper phase was collected, and the pellet was extracted three times with 3 ml each of the extraction medium. To the combined organic phases, a 6.7%, by weight, solution of potassium sulfate was added up to a volume of 47 ml. After vigorous shaking, the upper hexane-rich layer was removed. The organic phase which contained potentially oxylipins was subsequently dried under a nitrogen stream. The remaining lipids were redissolved in 1 ml of chloroform and stored under argon atmosphere at −80°C until use. At first, oxylipins were either isolated (hydroperoxy PUFA and hydroxy PUFA isomers) or analyzed (divinyl ethers) by reversed phase HPLC (RP-HPLC) on a Nucleosil C-18 column (Macherey-Nagel, KS-system, 250×4 mm, 5 mm particle size) with a solvent system of methanol/water/acetic acid (85/15/0.1, by volume) and a flow rate of 1 ml/min. For detection of the hydroperoxy PUFAs and hydroxy PUFAs, the absorbance at 234 nm indicating the conjugated diene system was recorded. For analysis of PUFAs, the absorbance at 210 nm indicating the double bond of the fatty acids was recorded [27]. For analysis of divinyl ethers, the absorbances at 250 nm and 268 nm indicating either one or two conjugated diene systems in conjugation to an ether bond of the fatty acids were recorded [28,29]. The different vinyl ether isomers eluted between 7.3 min (etherolenic acid) and 8.6 min (colneleic acid). Separation of hydroperoxy PUFA and hydroxy PUFA isomers was carried out by SP-HPLC on a Zorbax SIL column (HP, 250×4.6 mm, 5 mm particle size) with a solvent system of *n*-hexane/2-propanol/acetic acid (100/1/0.1, by volume) and a flow rate of 1 ml/min. The enantiomer composition of hydroperoxy PUFAs as well as of the hydroxy PUFAs was analyzed by chiral phase HPLC on a Chiralcel OD column (Diacel Chem. Industries, 250×4.6 mm, 5 mm particle size; purchased from Baker, Deventer, The Netherlands) with a solvent system of hexane/2-propanol/acetic acid (10/5/0.1, by volume) and a flow rate of 1 ml/min. All substances were identified by their characteristic UV spectra and by co-injection of authentic standards. Calibration curves (five point measurements) for LA, LeA, colneleic acid, etherolenic acid and 13-HOT were established.

The analysis of LOX-derived aldehydes from barley leaves was performed as described before using some modifications [26]. 1 g of frozen leaf segments was added to 10 ml of extraction buffer (methanol/2 mM HCl, 1/1, by volume, pH 3.0, with 0.001%, by weight, butylated hydroxytoluene), containing 20 ng of (2*E*)-pentenal as internal standard. The solution was immediately homogenized with an Ultra Turrax for 30 s under a stream of argon on ice. The extract was centrifuged at 4500×*g* at 4°C for 25 min. The clear upper phase was collected, and the pellet was extracted twice with 5 ml each of extraction buffer. In order to form the 2.4-dinitrophenylhydrazone (DNPH) derivatives of aldehydes, the collected upper phases were stirred in presence of 1 ml of DNPH reagent (2.4-dinitrophenylhydrazine, 0.1%, by weight, in 1 M HCl) at room temperature for 1 h. The reaction mixture was extracted three times with 5 ml hexane each, and the collected organic phases were dried under a stream of N_2. DNPH derivatives were redissolved in 400 μl of acetonitrile. HPLC analysis was carried out by RP-HPLC of the DNPH derivatives on a Jupiter C-18 300A column (250×1.0 mm, 5 μm particle size, Phenomenex, Germany) using a binary gradient system (solvent A: acetonitrile/water (60/40, by volume); solvent B: acetonitrile/water (80/20, by volume)) with the following gradient program: 100% solvent A for 15 min, followed by a linear increase of solvent B up to 50% solvent B within 5 min, by a linear increase of solvent B up to 100% within 13.4 min and finally by an isocratic post-run at 100% solvent B for 11.6 min. The flow rate was 0.05 ml/min. For analysis of the DNPH derivatives, the absorbance at 365 nm indicating the DNPH chromophore was recorded. All substances were identified by their characteristic UV spectra and by co-injection of authentic standards. Calibration curves (five point measurements) for all DNPH derivatives of the substances under investigation were established.

3. Results

3.1. SA-induced changes in the pattern of barley LOX forms

Barley leaves carry at least three LOXs of the molecular masses of 92, 98 and 100 kDa (LOX-92, LOX-98, LOX-100) which are differentially induced by JAME treatment [23]. In addition, for LOX-100, a transcriptional up-regulation by exogenous application of JAME has been shown [22]. Since SA induces the expression of LOX-100 as well [21], we compared the pattern of LOXs induced upon JAME and SA treatment at the protein level (Fig. 2). LOX-100 protein increased time-dependently upon JAME treatment and with some delay upon SA treatment, as expected by the similarly occurring transcriptional up-regulation [21]. In order to follow this question of a physiological function of the SA-induced LOXs, we recorded metabolic profiles of oxylipins upon SA

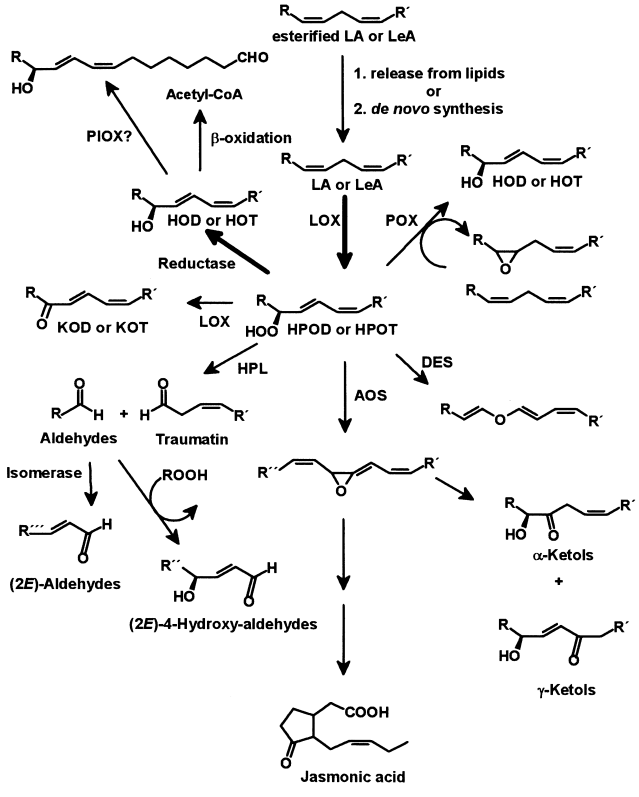

Fig. 1. Metabolic routes for LOX-dependent catabolism of PUFAs in plants. The preferential route described here for SA treatment of barley leaves is indicated by bold arrows.

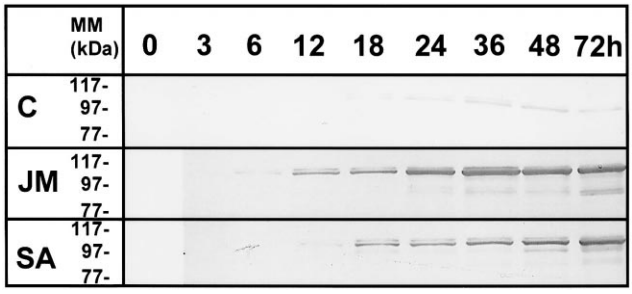

Fig. 2. Immunoblot analysis of LOX forms appearing in barley leaf segments upon water treatment (C), upon JAME treatment (JM) and upon SA treatment (SA).

treatment indicating the in vivo action of enzymes of the LOX pathway (Fig. 1).

3.2. Metabolic profiling of the LOX pathway

Both possible substrates of the LOX reaction in plants, LeA and LA, accumulated steadily up to 72 h, if leaf segments were floated on SA (Fig. 3A,B), whereas controls kept unchanged (water treatment). In addition, the 10-fold higher accumulation of LeA compared to LA reflects the usually occurring ratio between both fatty acids in leaf tissues [30]. The accumulation of the initial LOX products, either HPOD derived from LA or HPOT derived from LeA, was below the detection limit (data not shown). This indicated a remarkable flux of these central intermediates within the LOX pathway (Fig. 1). Most interestingly, formation of HOD and HOT, both indicative for the reductase branch in the LOX pathway, increased after 24 h of SA treatment to remarkable amounts (Fig. 3C,D). Again the 10-fold higher accumulation of HOT than that of HOD reflects the LeA/LA ratio of the tissue. Among the both positional isomers, a strong preponderance of the 13-isomers was observed. This reflects the specific induction of 13-LOXs as shown at least for LOX-100 [21,22].

Among the HPL-derived products, hexenal, (2*E*)-hexanal and (2*E*)-4-hydroxy-2-hexenal were identified recently as products of 13-HPOT and 13-HPOD in barley leaves [26]. Although they occurred at higher basic levels exhibiting an

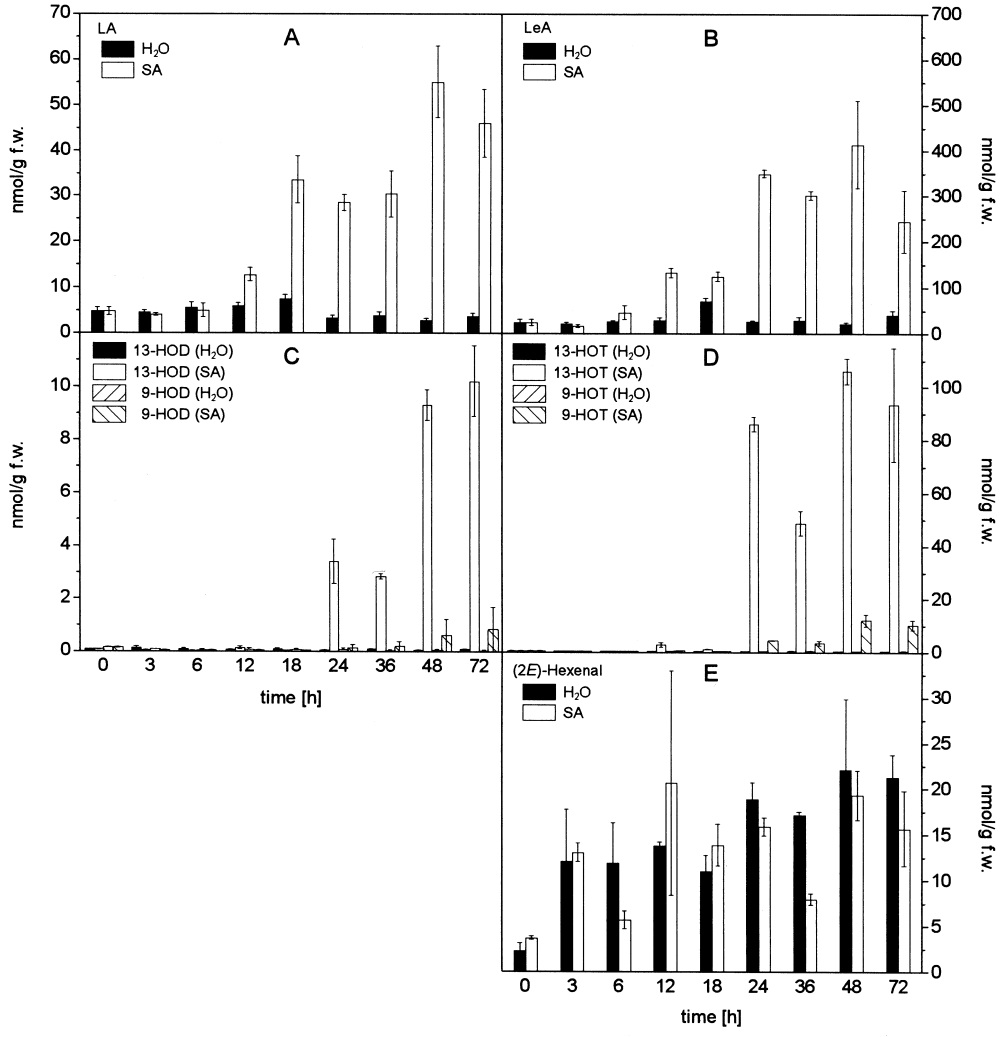

Fig. 3. Metabolic profiling of LA (A), LeA (B) and oxylipins derived therefrom (C–E) in barley leaf segments floated on water or 50 μM SA. As shown in C, upon SA treatment, the LA-derived LOX product is preferentially 13-HOD (open columns), whereas 9-HOD (hatched columns) accumulates much less. As shown in D, upon SA treatment, the LeA-derived LOX product is preferentially 13-HOT (open columns), whereas 9-HOT (hatched columns) accumulates much less. As indicated in E, SA treatment did not alter the level of leaf aldehydes such as (2*E*)-hexenal (white columns) compared to the water control.

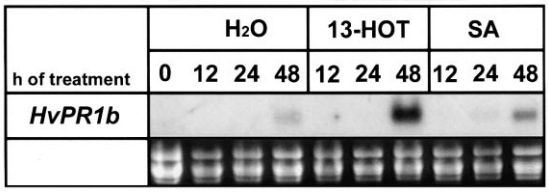

Fig. 4. Northern blot analysis of mRNA accumulation of *PR1b* upon treatment of barley leaf segments either with 10 μM 13-HOT or with 50 μM SA, respectively, for various times. Loading was checked by ethidium bromide staining (lower box).

about 2-fold rise by water treatment, there was no significant increase by SA treatment of all three compounds (only shown for (2*E*)-hexenal, Fig. 3E). Whereas JAME treatment led to the accumulation of leaf aldehydes [26], the data presented here indicate a remarkable shift of the LOX-derived products into the reductase-catalyzed reaction of the LOX pathway, if barley leaves were treated with SA. To test whether the DES branch is also more active upon SA treatment, we analyzed potential vinyl ether PUFAs. For all times of SA treatment, no vinyl ether PUFAs such as colneleic acid and etherolenic acid were detectable (data not shown).

3.3. 13-HOT induces PR1b expression

Expression of *PR1b* occurs upon infection of barley leaves with powdery mildew [21] or SA treatment (K.-H. Kogel, personal communication). Due to the accumulation of 13-HOT upon SA treatment, we analyzed whether this reductase-derived product leads to *PR1b* expression. As shown in Fig. 4, *PR1b* mRNA accumulated strongly after 48 h of 13-HOT treatment corresponding in time with accumulation by SA treatment.

4. Discussion

Various compounds of the LOX pathway are known to function in plant defense reactions. Whereas C_6 volatiles accumulate upon infection of bean leaves with *Pseudomonas syringae* pv. *phaseolicola* [4] and induce a subset of defense-related genes [31], divinyl ethers with antimicrobial activity were found to accumulate in blight-diseased potato leaves [6]. Most intriguing data are now available for JA. Beside its common function with ethylene as the major signals in the wound-response [32,33], for many plant-pathogen interactions, a signal transduction via the octadecanoid pathway has been demonstrated [34,35]. There are increasing data that jasmonate acts in a concerted manner with ethylene leading to defense-related gene products such as defensins [7] or thionines [34], whereas SA induces preferentially PR gene expression [11,12]. In addition to a SA-dependent pathway leading to SAR, a SA-independent pathway leading to the rhizobacteria-mediated ISR has been proposed [13]. In this pathway which is different from the defensin/thionine pathway and the wound-response pathway, JA and ethylene act also in a concerted manner [36]. Both the SAR and the ISR pathway have downstream from SA, ethylene and JA the regulatory protein NMR1 in common. These data may illustrate that the plant is adapted to the various environmental stimuli including differentially acting pathogens, by responding via a modular equipment of low molecular signals like JA, SA and ethylene and signalling proteins, respectively, with the expression of partially overlapping sets of genes [36].

Here, we add another facet. The SA-induced shift in oxylipin pattern may be of consequences for the defense response. On one side, antimicrobial activity of these compounds may occur [4], and on the other side, distinct sets of defense genes may be expressed as shown for C_6 volatiles [31]. Our data substantiate the recently proposed term 'oxylipin signature' as a distinct indicator of each plant [37]. This term was proposed by detecting specific oxylipin patterns derived from 18:3 and 16:3 fatty acids. Here, we show that a specific oxylipin pattern can occur by SA treatment as well. It remains to be elucidated whether this is a general feature of SA action or its specific property within a monocotyledonous plant. So far, several regulatory properties within the octadecanoid biosynthesis of barley, such as enzymatic properties and amino acid sequences of AOS and its transcriptional regulation by SA, were found to be different from dicotyledonous plants (H. Maucher and C. Wasternack, unpublished data). Further experiments will show whether the preferential formation of hydroxy fatty acids upon SA treatment is necessary and sufficient in the plant defense response or is a by-product of SA function.

The data presented here indicate for the first time the preferential activity of the reductase pathway among the various metabolic transformations of the LOX-derived 13-HPOT or 13-HPOD (Fig. 1). Whereas 13-HOT accumulated strongly upon SA treatment, no accumulation of products of the HPL (Fig. 3E), DES (data not shown) or AOS [21] could be detected. Both SA and 13-HOT accumulating by SA treatment are able to induce *PR1b* expression. This suggests that 13-HOT or metabolites derived therefrom may function in the establishment of SA-induced defense status. Till now, as physiological function of these metabolites, only their catabolism within the β-oxidation has been proposed [38]. However, an alternative catabolic pathway for 13-HOT has been described in MOLT4 lymphocytes [39,40]. Beside the degradation of 13-HOT by β-oxidation, the formation of the catabolite (12*S*,14*Z*,10*E*,8*Z*)-12-hydroxy-14,10,8-heptadecatrienal has been found. This compound might be formed by α-oxidation. After the recent identification of the pathogen-inducible PIOX as an α-oxidizing enzyme [41], it is tempting to speculate, that the formation of this aldehyde from 13-HOT might occur in barley leaves upon SA treatment as well. Although a physiological function for mammalian tissues has not been found, one may assume that it functions as membrane-permeable non-volatile defense compound in plants.

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