Hypothesis

Biotic and heavy metal stress response in plants: evidence for common signals

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Abstract In higher plants, biotic stress (e.g., herbivore or pathogen attack) as well as abiotic stress (in particular heavy metals) often induce the synthesis and accumulation of the same defense-related secondary metabolites. This well-known finding still awaits an explanation regarding the common features of both stress types. In this study, a mechanism is proposed that links reactive oxygen species (ROS) generation with lipid oxidation processes, ultimately resulting in the formation of similar, highly active signalling compounds. The generation of ROS is a common event in both heavy metal treatment and biotic stress although it can depend on quite different, enzymatic and non-enzymatic reactions. Regardless, ROS are involved in the oxidation of unsaturated fatty acids which initiate the formation of oxylipins, a highly variable class of lipid-derived compounds in plants. Oxylipins represent new endogenous signals involved in biotic- and abiotic-induced stress responses.

Keywords: Biotic stress; Heavy metal; Oxylipin; Plant secondary metabolite

1. Introduction

Plants possess biochemical defense mechanisms which prevent or reduce further damage from pathogens [1]. The defense includes the induction of both de novo biosynthesis and rapid accumulation of secondary metabolites, referred to as phytoalexins. These compounds represent organic molecules of low molecular weight that by definition are not required for normal physiological processes of the plant and, furthermore, exhibit antibiotic activities directly affecting a respective aggressor [2]. Due to this activity-based definition, phytoalexins are of high chemical diversity. Regardless of the plant species or taxon investigated, major classes of secondary metabolites are found, such as phenylpropanoids, terpenoids, and alkaloids [2]. Generally, phytoalexins are not induced by simple wounding but very commonly in plant–microbial pathogen interactions [1,2].

There are currently only few examples for phytoalexins involved in plant–herbivore interactions. Nevertheless, insect herbivore feeding often triggers the biosynthesis and release of certain plant secondary compounds, volatile semiochemicals, which attract natural enemies of the feeding insect [3]. Typical volatiles are represented by mono- and sesquiterpenoids, oxidized fatty acid derivatives, and aromatics [3,4]. In all cases, the biosynthesis of these secondary compounds is highly regulated by the plant, economizing on processes which are not constitutively necessary. Consequently, control is exercised in terms of pest recognition, intra- and intercellular signalling, and finally the onset of localized and systemic defense responses. Surprisingly, there are many examples available from the literature describing plants which synthesize and accumulate secondary metabolites upon treatment with an abiotic stress factor, mainly heavy metal ions (Table 1). All of the compounds listed have been described as phytoalexins of the particular species, typically induced during pathogen attack. Well-known examples are glyceollins, representing isoflavonoid phytoalexins involved in the interaction between soybean (*Glycine max* L.) and the phytopathogenic oomycete, *Phytophthora sojae*, one of the best studied pathosystems at all [5]. In 1980, Moesta and Grisebach [6] were able to induce the biosynthesis of glyceollins in soybean simply by mercury ($HgCl_2$) treatment. Up to now, there is no convincing explanation for this finding or for any other of the examples given in Table 1.

Because phytoalexins represent a biosynthetically diverse group and the same type of cellular reaction in the form of induction of secondary metabolism takes place in different unrelated plant species, the underlying molecular and biochemical mechanisms are very likely general. In this hypothesis, we propose a mechanism linking oxidative stress reactions, common in both heavy metal and pathogen challenge, and lipid oxidation processes generating oxylipins. These lipid-derived compounds have been identified very recently as signalling molecules in plants elicited by pathogens and are probably also responsible for heavy metal-induced defense responses as well. Current and previously published data are presented below to support this hypothesis.

2. Enzymatic and non-enzymatic sources for reactive oxygen species in plant cells

2.1. Heavy metal-induced oxidative stress

Reactive oxygen species (ROS) such as $\cdot O_2^-$, H_2O_2, and $\cdot OH$ are commonly generated under stress conditions and bear strong oxidizing activities that can attack all types of biomolecules [7]. In fact, these oxygen species represent intermediates

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Table 1
Survey of heavy metal treatment-induced secondary metabolite accumulation in various plant species

Species	Secondary metabolites	Inductor	Reference
Glycine max	Glyceollins, THP	$HgCl_2$	[6]
Lupinus albus	Genistein[a], 2-OH-genistein[a]	$CuCl_2$	[31]
Medicago sativa	Medicarpin, vestitol, sativan; MGM, FGM	$CuCl_2$	[32,33]
Pisum sativum	Pisatin	$CuCl_2$	[34]
Trifolium repens	Medicarpin	$HgCl_2$	[35]
Trifolium pratense	(−)-Maakiain, formononetin	$CuCl_2$	[36]
Brassica sp.	Indole phytoalexins	$CuCl_2$	[37]
Chamomilla recutita	Umbelliferone	$CuCl_2$	[38]
Daucus carota	6-Methoxymellein	$CuCl_2$; $HgCl_2$	[39,40]
Sorbus aucuparia	Aucuparin	$CuCl_2$	[41]
Helianthus tuberosus	7-OH-Coumarines	$CuCl_2$	[42]
Datura stramonium	Lubimin, 3-OH-lubimin	$CuCl_2$; $CdCl_2$	[43]
Oryza sativa	Sakuranetin; volatiles	$CuCl_2$	[44,45]
Zea mays	HDMBOA-Glc	$CuCl_2$	[46]

THP, trihydroxypterocarpan; MGM, medicarpin-3-*O*-glucosid-6″-*O*-malonate; FGM, formononetin-7-*O*-glucosid-6″-*O*-malonate; HDMBOA-Glc, 2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose; volatiles: methylsalicylate, monoterpenes, sesquiterpenes, (*Z*)-3-hexen-3-ol.
[a] Prenylated.

emerging during the successive reduction of O_2 to H_2O. Plants' exposure to certain heavy metal ions shifts the balance of free radical metabolism towards an accumulation of H_2O_2. In the presence of redox active transition metals such as Cu^+ and Fe^{2+}, H_2O_2 can be converted to the highly reactive ·OH molecule in a metal-catalyzed reaction via the Fenton reaction. The oxidized metal ions undergo a re-reduction in a subsequent reaction with superoxide radicals ($\cdot O_2^-$) (Scheme 1). An alternative mechanism of ·OH formation directly from H_2O_2 and $\cdot O_2^-$ is the metal-independent Haber–Weiss reaction (Scheme 1).

Fenton reaction:

$$H_2O_2 + Fe^{2+}/Cu^+ \rightarrow \cdot OH + OH^- + Fe^{3+}/Cu^{2+}$$

$$\cdot O_2^- + Fe^{3+}/Cu^{2+} \rightarrow Fe^{2+}/Cu^+ + O_2$$

Haber–Weiss reaction:

$$H_2O_2 + {}^{\bullet}O_2^- \rightarrow {}^{\bullet}OH + OH^- + O_2$$

Scheme 1. Chemical reactions involved in hydroxyl radical (·OH) generation.

The ·OH molecule is one of the most reactive species known. Because of its ability to initiate radical chain reactions it is very likely responsible for irreversible chemical modifications of various cellular components. Another ROS that might be involved mainly in lipid peroxidation is the protonated form of $\cdot O_2^-$, the hydroperoxyl radical ($\cdot O_2H$). These species exist in equilibrium. As Hg^{2+} does not belong to the transition metals, it cannot replace Cu^+ and Fe^{2+} in the Fenton reaction. This calls for a different mechanism that causes an accumulation of ROS. Possible explanations are that Hg^{2+} ions inhibit the activities of antioxidative enzymes especially of glutathione reductase, and also raise a transient depletion of GSH [8]. Thus, a natural accumulation of ROS would be the consequence.

2.2. Pathogen/herbivore-induced oxidative stress

Besides intracellular sources of ROS that include mitochondria, chloroplasts, and peroxisomes, the inducible production and accumulation of ROS in plants as a defense response to pathogen attack is very well documented and described as oxidative burst [7]. The ROS that have been detected in plant pathogen interactions are $\cdot O_2^-$ ($\cdot O_2H$), H_2O_2, and ·OH [9]. Obviously, the same ROS are present as during heavy metal stress. However, the enzymatic origin of these inducible ROS is still under discussion; various potential sources have been described in different plant species. These include apoplastic amine, diamine, and polyamine oxidase-type enzymes [10], a cell wall localized peroxidase that directly forms H_2O_2 [11], and a plasma membrane localized NADPH oxidase [12]. This latter enzyme represents the most widely studied mechanism for the synthesis of ROS (for details, see [9]). The product of this NADPH oxidase activity is very likely $\cdot O_2^-$, which is converted to the more stable ROS forms of H_2O_2 and O_2 spontaneously or by a superoxide dismutase reaction.

In the case of herbivory, the origin of ROS is not that clear. However, insect feeding causes wounding and thus the production of ROS in the damaged tissue [4,13]. As shown for soybean, herbivory by the insect *Helicoverpa tea* induced a shift in the oxidative status of the plant causing an increase in $\cdot O_2^-$ and ·OH radical formation [14]. In a more direct way, insect salivary gland-derived enzymes such as the H_2O_2 generating glucose oxidase might contribute to the increase in the concentrations of ROS at the side of herbivore attack [15].

3. Lipid peroxidation and formation of oxylipins

The main cellular components that are susceptible to damage by free radicals are proteins, DNA, carbohydrates, and lipids. ROS action on cell membrane results in the natural metabolic process of peroxidation of polyunsaturated fatty acids (PUFA) in membrane lipids, a non-enzymatic reaction initiated especially by the most reactive oxygen species, ·OH, and $\cdot O_2H$ which is more lipophilic than its non-protonated form, $\cdot O_2^-$, and thus able to penetrate the membranes more easily [7]. Enzymatic lipid peroxidation processes in plants catalyzed by enzymes, such as an α-dioxygenase, peroxidases or lipoxygenases (LOX), can convert unsaturated fatty acids to lipid peroxides as well [16, and references therein]. The main PUFA present in higher plants are linoleic acid (C18:2) and linolenic acid (C18:3). These are the main substrates of plant

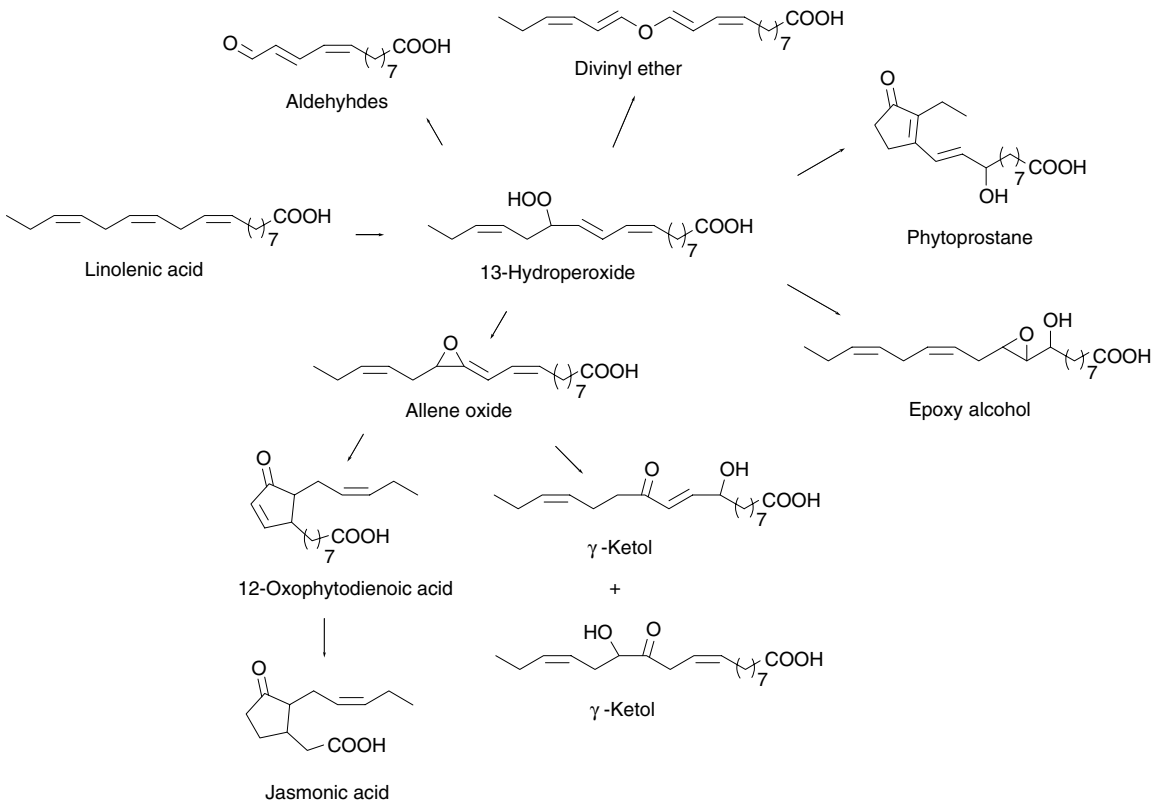

Fig. 1. Structures of various active plant oxylipins derived from the PUFA, linolenic acid (C18:3).

LOX, non-heme iron-containing fatty acid dioxygenases, which catalyze the oxidation of unsaturated C18 fatty acids into either 9- or 13-hydroxyperoxides, or a mixture of both, depending on the enzyme. Thus, both the products of LOX activities as well as the products of non-enzymatic reactions described above can form hydroperoxyoctadecadi(tri)enoic acids. Subsequently, a diverse array of enzymatic modifications is present in the plant leading to the generation of large numbers of structurally different oxylipins, i.e., oxidized metabolites of (unsaturated) fatty acids (Fig. 1) [16–18, and references therein]. Certainly, these pathways are independent on the origin of the hydroperoxide derivatives of the fatty acids. Moreover, Mueller and co-workers [19,20] showed in a remarkable series of studies that in plants a solely free radical-catalyzed oxidation of linolenic acid yielded a high number of phytoprostanes, isomeric oxylipins of a cyclic C18-isoprostane type (Fig. 1). Interestingly, F_1-phytoprostan levels have been induced by the presence of heavy metals and wounding, respectively. Both are treatments known to induce the generation of ROS [19].

4. A role for oxylipins in plant signalling

Many oxylipins, in particular those belonging to the jasmonate family (Fig. 1), are discussed as general inter- and intracellular signalling compounds involved in multiple defense reactions in response to pathogen and herbivore attack. For example, elicitations of proteinase inhibitors, volatile compounds, secondary metabolites, and defense genes have been reported [16,21–23]. Jasmonates and their biosynthetic C18 precursors, octadecanoids, are ubiquitously occurring linolenic acid-derived oxylipins. Different octadecanoids clearly vary in their abilities to influence plant responses. Results supporting this finding include either the induction of phytoalexins in soybean cell cultures or the composition of volatiles, emitted after treatment of Lima bean (*Phaseolus lunatus* L.) leaves. In soybean, the biosynthetic precursor of the jasmonates, 12-oxophytodienoic acid, was an active inducer of glyceollin synthesis, in contrast to jasmonic acid [24]. In Lima beans, early intermediates of the jasmonate biosynthesis (linolenic acid, 12-oxo-phytodienoic acid) elicited the biosynthesis of a homoterpene of diterpenoid origin whereas the final product of this pathway, especially jasmonic acid, triggered the synthesis of mono- and sesquiterpenes [25]. In addition to the jasmonates, other oxylipins such as epoxy, hydroxy, and divinyl ether derivatives (Fig. 1), which are also derived from PUFA hydroperoxides (PUFA-OOH), have been described to be associated with plant defense responses to pathogens and herbivores [16,18]. In addition, the enzyme-independent formation of phytoprostans strongly extended the number of oxylipins exhibiting a high biological activity in terms of inducing defense responses in planta, including the synthesis of secondary metabolites [20]. Interestingly, upon exogenous application 12-oxo-phytodienoic acid triggered glyceollin biosynthesis and accumulation in soybean, although during pathogen elicitor-treatment no increase of 12-oxo-phytodienoic acid has been detected [24]. This result indicated that some oxylipins having a certain biological activity to elicit a response must not necessarily be involved in all physiological processes leading to the onset of this particular response. Thus, it is conceivable that either (i) at least two independent signalling pathways exist

leading to the same result or (ii) the added oxylipin is structurally related to other still unknown oxylipins which indeed are involved in signalling processes. For the example just described, candidates for such compounds might be fatty acid metabolites which contain electrophilic α,β-unsaturated carbonyl groups as structural feature as suggested by Farmer and colleagues (Fig. 1) [21,26]. Remarkably, 12-oxo-phytodienoic acid belongs to this type of oxylipins.

5. ROS and fatty acids: a simple cocktail generates signals

Lipid-based signalling and their relationship to plant defense against mechanical stress, pathogens and herbivores was highly acknowledged during the last years and is still a field of increasing interest. The oxylipin group of fatty acid derivatives obviously plays a pivotal role as a source for signalling compounds in abiotic and biotic stress reactions. Although pathogen infections, herbivore attacks or even heavy metal treatment are quite different forms of stress, plants show a common response: an induced synthesis and accumulation of secondary compounds. Here, for this finding an underlying mechanism is proposed that is as simple as efficient. The common feature is the ROS-mediated or/and enzyme-catalyzed formation of fatty acid hydroperoxides. The presence of these compounds is a prerequisite and the initial point for the spontaneous or enzyme-catalyzed generation of numerous oxylipins. Very likely, many oxylipins have been neither identified yet nor characterized in terms of their biological activities in induction of plant secondary metabolism. However, given the large number of structurally different oxylipins, it is highly probable that these compounds represent a pool of active signalling molecules that contribute to the plasticity of (defense) responses in plants. Due to their structures and multiple biological activities, the role of oxylipins in plants is comparable to that of eicosanoids in animals. The variety of biological responses that are inducible by oxylipins might be mediated by defined structure–activity relationships. However, structurally related oxylipins could have overlapping activities in their capacity to induce the same set of responses [21], possibly with different threshold concentrations.

Thus, it is tempting to speculate that a non-regulated formation of oxylipins initiated by the presence of heavy metals might elicit plant secondary metabolism by the generation of structurally similar or even identical compounds that are involved coincidentally in defense reactions directed against biotic challenges (Fig. 2). To support this hypothesis, Lima beans have been treated with copper to investigate the induction of secondary metabolism with the focus on volatile compounds well known from herbivore infestation [27]. The result shows that this simple treatment was sufficient to elicit at least in part the biosynthesis of secondary metabolites which among other volatiles are described for herbivory-induced defense responses (Fig. 3). In fact, the copper-induced volatile blend exactly resembles the "bouquet" obtained upon treatment with an ion channel-forming peptide antibiotic, alamethicin, used as elicitor in Lima bean [28]. Although the mechanism of how alamethicin induces volatile synthesis is not clear, for another ion channel-forming antibiotic, amphotericin B, its ability to cause ROS production in plant cells has been demonstrated [29], suggesting a similar mechanism for

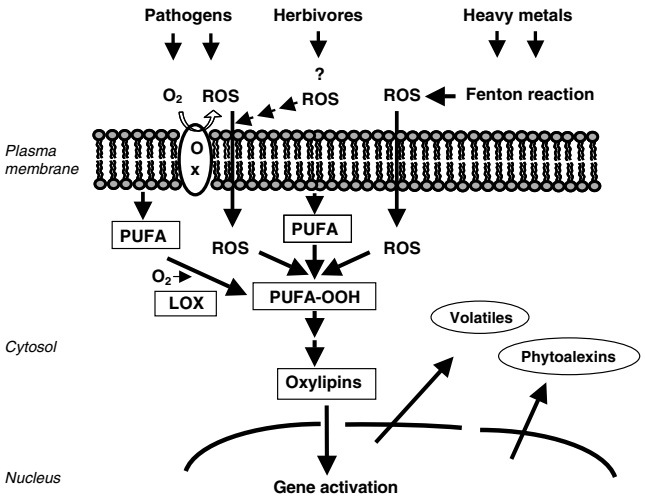

Fig. 2. Model for biotic and heavy metal treatment-induced biosynthesis of secondary metabolites. ROS originate either from enzyme activities such as NADPH oxidase (Ox) or spontaneously. ROS or LOX oxidize PUFA to PUFA-OOH which are converted enzymatically or non-enzymatically to oxylipins. Oxylipins on their part induce expression of genes involved in the biosynthesis and accumulation of secondary metabolites. For clarity, the model simplifies herbivore- and pathogen-elicited processes by not including receptor-mediated signalling events such as elicitor–receptor interactions, cytosolic calcium transients, or protein phosphorylation/dephosphorylation cascades [1,4,5,13].

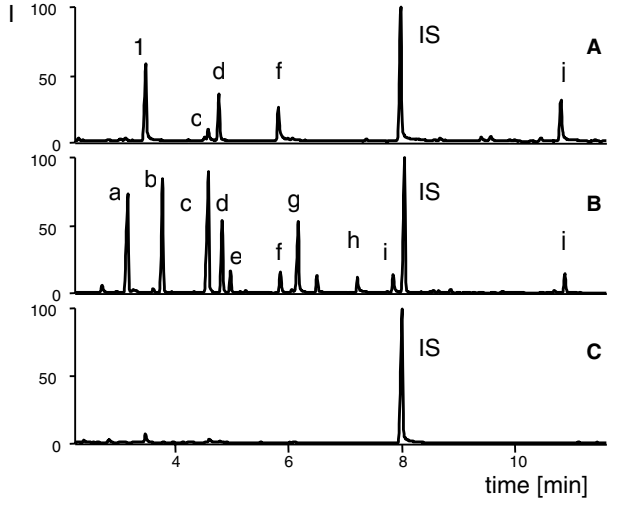

Fig. 3. Gas chromatographic profiles of volatiles from Lima bean leaves (*Phaseolus lunatus* L.). Plantles were treated for 24 h with 700 μM $CuSO_4$ in water (A), with 6 *Spodoptera littoralis* larvae (B), and with water as control (C). After collection of the volatiles compounds were separated and identified by combined GC/MS as described [24]. Identification of compounds: (a) (3*Z*)-hexenylacetate, (b) β-ocimene, (c) linalool, (d) 4,8-dimethylnona-1,3,7-triene, (e) $C_{10}H_{14}$, (f) methyl salicylate, (g) $C_{10}H_{16}O$, (h) indole, (i) methylanthranil, (j) 4,8,12-trimethyltrideca-1,3,7,11-tetraene, (1) ethylhexanol (contamination). IS, internal standard: 1-bromodecane.

alamethicin. Moreover, in the presence of aristolochic acid (0.3 mM), a phospholipase A_2 inhibitor that prevents the release of fatty acids from phospholipids [30], no volatile emission was detected upon copper and alamethicin treatment, supporting the involvement of free fatty acids as precursor in the induction process.

Although the hypothesis presented in this article needs more experimental confirmation, the concept of ROS and unsaturated fatty acid-derived signals provides a reasonable explanation for the similarities in heavy metal and certain biotic stress responses in plants. However, the data available indicate that abiotic stress such as heavy metals or membrane integrity-disturbing compounds can only partially mimic biotic interactions with respect to the activation of secondary metabolism initialized by ROS generation. In the near future, the pool of oxylipins available in plants under various physiological conditions has to be identified and determined regarding their structures, and their synthesis. Genetic analysis in addition to physiological studies will be useful to position oxylipin signals in the transduction pathways and to understand how these signals are perceived and mediated to downstream responses. Transcriptional profiling techniques such as cDNA microarrays will allow studies on how the expression of biosynthetic genes of interest varies in response to oxylipins and/or heavy metals. Labelled fatty acid precursors might represent valuable tools for the elucidation of lipid peroxidation processes. Plant mutants that are unable to synthesize or release some of the PUFA involved in oxidative lipid metabolism could be helpful to investigate the biosynthetic pathway of oxylipins. Finally, the chemical synthesis of oxylipins might help to generate sufficient amounts of oxylipins to analyze their definitive biological activities, to learn more about their functions and make use of it.

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