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Cyclopentenone isoprostanes induced by reactive oxygen species trigger defense gene activation and phytoalexin accumulation in plants

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Summary

Lipid peroxidation may be initiated either by lipoxygenases or by reactive oxygen species (ROS). Enzymatic oxidation of α-linolenate can result in the biosynthesis of cyclic oxylipins of the jasmonate type while free-radical-catalyzed oxidation of α-linolenate may yield several classes of cyclic oxylipins termed phytoprostanes *in vivo*. Previously, we have shown that one of these classes, the E_1-phytoprostanes (PPE_1), occurs ubiquitously in plants. In this work, it is shown that PPE_1 are converted to novel cyclopentenone A_1- and B_1-phytoprostanes (PPA_1 and PPB_1) *in planta*. Enhanced formation of PPE_1, PPA_1, and PPB_1 is observed after peroxide stress in tobacco cell cultures as well as after infection of tomato plants with a necrotrophic fungus, *Botrytis cinerea*. PPA_1 and PPB_1 display powerful biologic activities including activation of mitogen-activated protein kinase (MAPK) and induction of glutathione-S-transferase (GST), defense genes, and phytoalexins. Data collected so far infer that enhanced phytoprostane formation is a general consequence of oxidative stress in plants. We propose that phytoprostanes are components of an oxidant-injury-sensing, archaic signaling system that serves to induce several plant defense mechanisms.

Keywords: isoprostane, jasmonic acid, phytoprostane, prostaglandin, 12-oxo-phytodienoic.

Introduction

In the early 1990s, a series of prostaglandin (PG)-like compounds termed isoprostanes were discovered to be produced from arachidonic acid by a free-radical-catalyzed mechanism independent of the cyclooxygenase enzyme in humans (Morrow *et al.*, 1990; Roberts and Morrow, 1997). Currently, isoprostanes are widely used as biomarkers of lipid peroxidation in mammals, and elevated levels of isoprostanes have been reported in a variety of diseases associated with excessive generation of free radicals (Lawson *et al.*, 1999; Mueller, 1998). Moreover, several isoprostanes have been shown to display a variety of potent biologic activities, including vasoconstriction, smooth muscle contraction, platelet aggregation/adhesion, neurovascular and pulmonary endothelial cytotoxicity, as well as induction of genes, notably of cyclooxygenase-2 in the low nanomolar concentration range, and thus are discussed as mediators of oxidative stress in animals (Cracowski *et al.*, 2001; Janssen, 2001; Roberts and Morrow, 2002).

Higher plants do not have the enzymatic capacity to form arachidonate (C20 : 4), and hence lack the mammalian C20 pathways to synthesize prostaglandins or C20-isoprostanes. Instead, one of the major unsaturated fatty acids in plants, α-linolenic acid (C18 : 3), can be used for enzymatic synthesis of prostaglandin-like compounds, such as jasmonic acid (Gundlach *et al.*, 1992; Parchmann *et al.*, 1997), and for non-enzymatic synthesis of several classes of C18-isoprostanes (dinor isoprostanes) termed phytoprostanes (PP) (Imbusch and Mueller, 2000a,b; Parchmann and Mueller, 1998). The biochemical reaction sequence leading to five of these classes, PPG_1, PPE_1, PPA_1, PPB_1, and PPF_1, is shown in Figure 1. Two phytoprostane regioisomers (types I and II) of each class can be formed, each of which comprises several isomers depending on the prostanoid ring system (Figure 1).

Recently, E_1- and F_1-phytoprostanes (PPE_1 and PPF_1) have been found to occur apparently ubiquitously in higher

plants at basal levels (Imbusch and Mueller, 2000a; Parchmann and Mueller, 1998) that are in the range of their enzymatically synthesized congeners, 12-oxo-phytodienoic acid (12-OPDA) and jasmonic acid (JA). Moreover, F_1-phytoprostane levels can be dramatically induced by oxidative stress (peroxides, heavy metals, wounding) *in planta* (Imbusch and Mueller, 2000b) and may exceed levels of jasmonates in maximally elicited plant cells by more than an order of magnitude (Imbusch and Mueller, 2000b; Parchmann *et al.*, 1997).

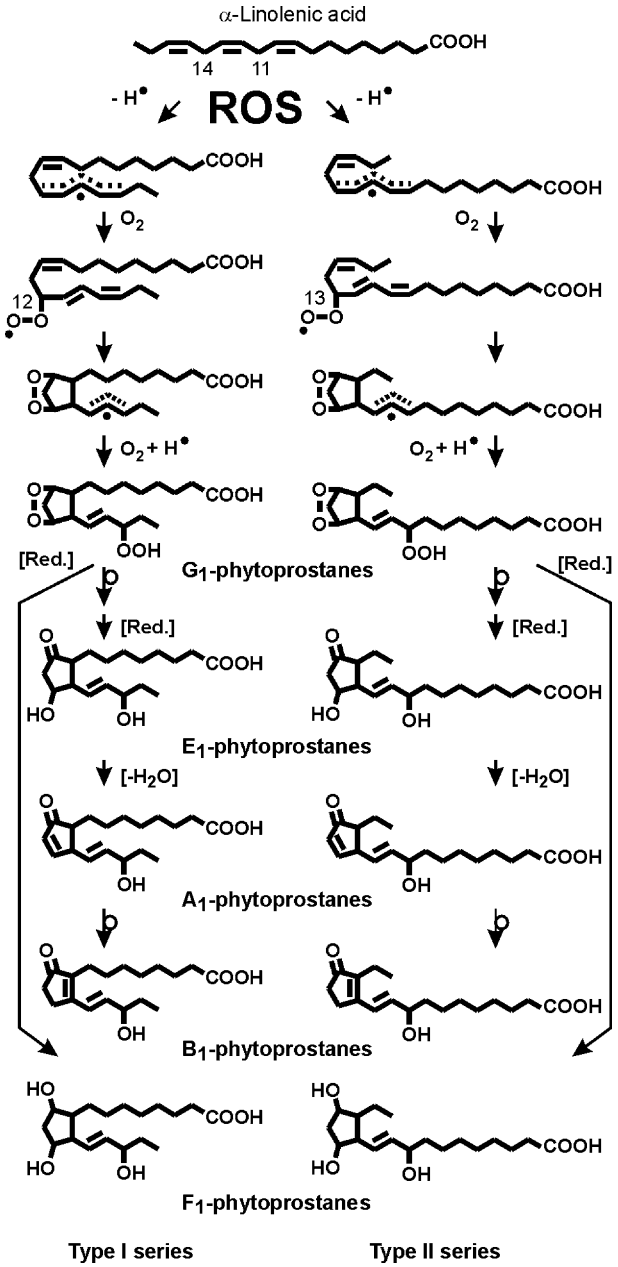

Figure 1. Formation of phytoprostanes.
Non-enzymatic, free-radical-catalyzed oxygenation of linolenic acid yields two series of regioisomeric PPG_1 (types I and II) that may decompose to PPE_1 and PPF_1. PPE_1 are prone to undergo spontaneous dehydration/isomerization to yield PPA_1 and PPB_1. The letters behind the acronym PP (phytoprostanes) qualify the ring system, and the numerical subscript indicates the number of double bonds in the side chains according to the general isoprostane/prostaglandin nomenclature (Rokach *et al.*, 1997). All products are racemic, i.e. each regioisomer of PPG_1, PPF_1, and PPE_1 is theoretically composed of 16 isomers, while each regioisomer of PPA_1 and PPB_1 comprises of eight and two isomers, respectively.

It is of paramount importance to clarify whether or not phytoprostanes generated during oxidative stress in plants are biologically active as it has been demonstrated for isoprostanes in animals. For mammalian isoprostanes, it has been shown that several isoprostanes bind to and activate receptors of their enzymatically synthesized prostaglandin congeners. However, some isoprostanes may also induce enzymatic prostaglandin synthesis (Janssen, 2001). As plant isoprostanes do also have their enzymatically synthesized congeners, the jasmonates, we speculated that phytoprostanes may display actions similar to those of jasmonates. To this end, we measured oxylipin profiles (jasmonates, phytoprostanes) in plant cell cultures and plants suffering from oxidative stress. We also compared phytoprostane and jasmonate activities in a variety of well-established bioassays.

In this study, we show that peroxide stress in tobacco cell cultures as well as infection of tomato plants with the necrotrophic fungus *Botrytis cinerea* induces levels of endogenous PPE_1 as well as levels of previously unidentified cyclopentenone phytoprostanes, PPA_1 and PPB_1. We also show that cyclopentenone A_1- and B_1-phytoprostanes display a variety of biologic activities, including induction of glutathione-S-transferase (*GST*), phytoalexin biosynthesis, as well as induction of genes involved in primary and secondary metabolism. Moreover, rapid activation of mitogen-activated protein kinase (MAPK) activity by phytoprostanes was observed. Thus, evidence obtained supports the concept that isoprostanes play a role in oxidative stress signaling, not only in animals but also in plants.

Results

Cyclopentenone A_1- and B_1-phytoprostanes occur endogenously in plants – chemical synthesis, structure elucidation, and analysis

Several cyclopentanone and cyclopentenone fatty acids structurally related to jasmonates can be formed via the phytoprostane pathway in plants. One branch of this pathway potentially yields E_1-, A_1- and B_1-phytoprostanes from which only PPE_1 have been identified in plants previously (Parchmann and Mueller, 1998). Novel cyclopentenone A_1- and B_1-phytoprostanes were prepared from *in vitro* autoxidized linolenate in order to develop analytical procedures for quantification of endogenous levels of these compounds in plants, as well as to study their biologic

properties. Partially *in vitro* auto-oxidized linolenate contained cyclopentenones, PPA_1 and PPB_1, in addition to the previously described PPE_1. However, it proved to be difficult to purify cyclopentenones from the complex auto-oxidation mixture directly. Therefore, PPE_1 were isolated from partially auto-oxidized α-linolenic acid (Parchmann and Mueller, 1998), purified and converted to PPA_1 and PPB_1 using standard chemical methods well known in prostaglandin chemistry. Chemically prepared cyclopentenone phytoprostanes, PPA_1 and PPB_1, were characterized by gas chromatography–mass spectrometry (GC–MS, Figure 2) as well as by nuclear magnetic resonance spectroscopy (NMR) and quantified by high-pressure liquid chromatography (HPLC) (see Experimental procedures for details).

A highly sensitive negative ion chemical ionization (NCI)-GC–MS method was developed for quantification of PPA_1 and PPB_1 in plant tissues. Prior to work-up, plant leaves were treated with [^{18}O]PPB_1 (internal standard). PPA_1 and PPB_1 were extracted, purified by solid-phase extraction and analyzed as their corresponding pentafluorobenzyl ester, trimethylsilyl ethers by NCI-GC–MS. As PPA_1 isomerized almost quantitatively to PPB_1 during the derivatization procedure and/or in the GC injector, both phytoprostane classes were collectively detected and quantified as PPB_1. PPA_1/B_1 were present in all the seven plant species so far analyzed at levels ranging from 11 to 131 ng g^{-1} of DW (Table 1). A representative GC–MS chromatogram is shown

Table 1 A_1/B_1-phytoprostane levels in plant leaves and cell cultures

	PPA_1/B_1, ng g^{-1} of DW	
	Type I	Type II
Arabidopsis thaliana	62.9 ± 11.5	68.4 ± 11.8
Betula pendula	8.9 ± 4.4	5.8 ± 1.0
Nicotiana tabacum	24.8 ± 9.6	28.4 ± 12.8
Lycopersicon esculentum	11.2 ± 5.7	5.7 ± 2.3
Tilia cordata	10.4 ± 1.9	8.3 ± 2.4
Salix alba	5.9 ± 2.7	4.7 ± 2.3
Rauvolfia serpentina	9.6 ± 1.8	10.9 ± 2.1 (cell culture)

Phytoprostane levels were determined as described in Experimental procedures. Results are expressed as mean ± SD (n = 3–6).

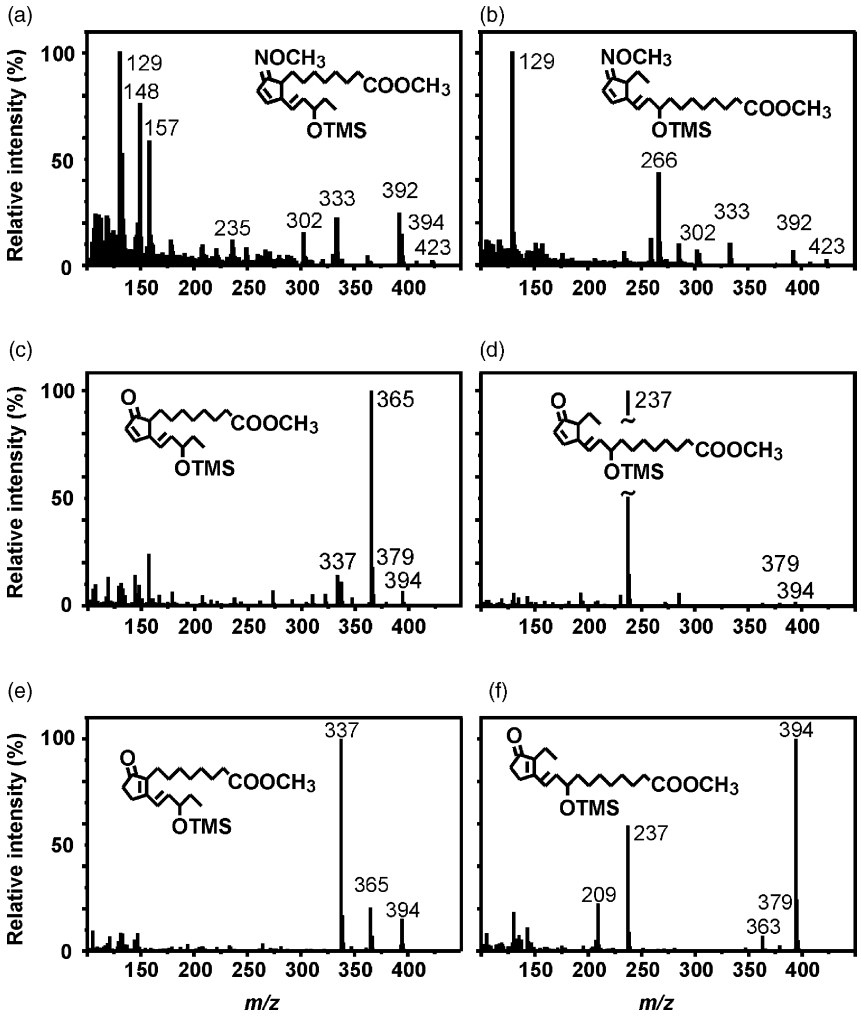

Figure 2. GC-EI-MS spectra of cyclopentenone phytoprostane derivatives.
Spectra of derivatives of PPA_1 type I (a,c), PPA_1 type II (b,c), PPB_1 type I (e), and PPB_1 type II (f) are shown.

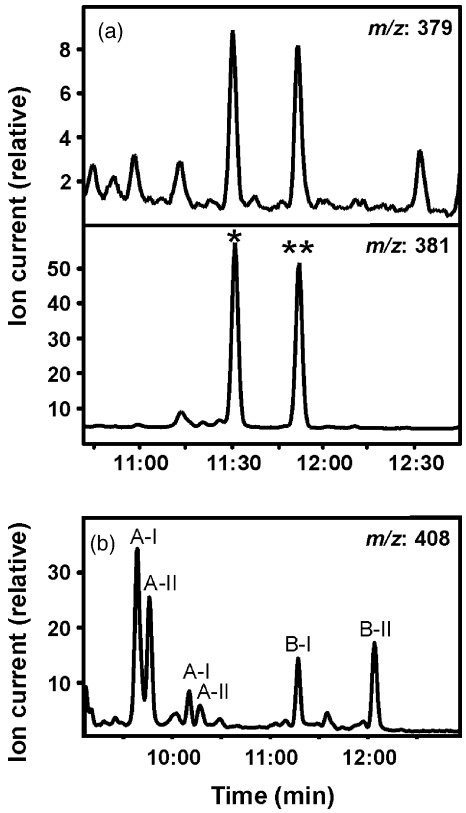

Figure 3. Representative selected ion monitoring GC-NCI-MS traces of A_1- and B_1-phytoprostanes (types I and II) from tomato leaves.
(a) PPA_1/B_1 were extracted from fresh tomato leaves and analyzed as their corresponding pentafluorobenzyl ester, trimethylsilyl ether PPB_1 derivatives. Two peaks are present in the *m/z* 379 ion current chromatogram, representing endogenous PPA_1/B_1 regioisomers of type I (15.8 ng) and type II (13.6 ng). The *m/z* 381 chromatogram displays the $[^{18}O]PPB_1$ peaks (*, type I; **, type II) of the internal standard (100 ng of each regioisomer).
(b) PPA_1 and PPB_1 were extracted from fresh tomato leaves and analyzed as their corresponding pentafluorobenzyl ester, trimethylsilyl ether, methoxime derivatives. The *m/z* 408 chromatogram shows peaks corresponding to PPA_1 type I (A-I), PPA_1 type II (A-II), PPB_1 type I (B-I), and PPB_1 type II (B-II).

in Figure 3(a). In order to determine PPA_1 and PPB_1 levels separately, analytical derivatization of the carbonyl groups to their corresponding methoximes is necessary to prevent isomerization of PPA_1 to PPB_1. As methoximation replaces the oxygen-18 label located in the cyclopentenone ring of the internal standard by the methoxime group, this method could not be used for direct quantification of PPA_1 and PPB_1. However, this derivatization procedure could be used to determine the endogenous ratio of PPA_1 to PPB_1 as shown in Figure 3(b). From the combined information of the two experiments, the levels of PPA_1 and PPB_1 could be calculated. In leaves of *Lycopersicon esculentum* (cv. Moneymaker), the endogenous ratio of PPA_1 to PPB_1 was 74 : 26 (12.5 ng g^{-1} PPA_1 and 4.4 ng g^{-1} PPB_1) and in leaves of *Nicotiana tabacum* (cv. Xanthi), a similar ratio of 70 : 30 for PPA_1 and PPB_1 (37.2 ng g^{-1} PPA_1 and 16.0 ng g^{-1} PPB_1) was found. Interestingly, basal levels of non-enzymatically formed phytoprostanes are in the concentration range of enzymatically formed jasmonic acid. Results suggest that non-enzymatic lipid peroxidation is an ongoing process even in healthy, untreated plants, which delivers significant amounts of cyclic fatty acids structurally related to jasmonates.

Peroxides induce transient E_1-, A_1-, and B_1-phytoprostane formation in tobacco cell cultures but do not trigger the jasmonate pathway

Previously, it was shown that the levels of PPF_1 could be dramatically induced by peroxides, copper ions, and wounding in different plant species (Imbusch and Mueller, 2000b). As both PPE_1 and PPF_1 are derived from the unstable PPG_1 (Figure 1) which is formed by free-radical-catalyzed cyclization of α-linolenate (Imbusch and Mueller, 2000a; Parchmann and Mueller, 1998), it is likely that all phytoprostane classes are induced by oxidative stress simultaneously. Indeed, treatment of tobacco cell cultures with 1 mM *tert*-butyl hydroperoxide (a catalase-resistant hydrogen peroxide analog that yields free radicals in the presence of reactive metals such as iron or copper ions) resulted in a rapid and dramatic increase in PPE_1 levels. Levels of PPE_1 types I and II increased 3- and 10.7-fold, respectively, reached a maximum after 1 h (60 and 215 ng g^{-1} of DW, respectively) and declined to almost baseline levels in the following 2 h (Figure 4a,b). Apparently, both PPE_1 regioisomers were rapidly metabolized or degraded *in vivo*.

Moreover, levels of PPA_1/B_1 types I and II increased eightfold reaching a first maximum (23.5 and 23 ng g^{-1} of DW, respectively) 1 h after addition of butyl hydroperoxide (Figure 4c,d). Levels of PPA_1/B_1 followed a biphasic time course with a second maximum after 120 min and close to baseline levels after 90 and 180 min.

While non-enzymatically formed phytoprostanes were dramatically induced by butyl hydroperoxide, the jasmonic acid pathway was not triggered. Jasmonic acid levels remained constant and were below the concentrations of PPE_1 and PPA_1/B_2 (Figure 4e) at all time points during the experiment.

Peroxides induce scopoletin accumulation in the medium of tobacco cell cultures

Reactive oxygen species (ROS) initiate lipid peroxidation (Figure 4a–d) but may also induce defense genes and phytoalexin synthesis in various species (Jabs *et al.*, 1997; Tierens *et al.*, 2002; Wojtaszek, 1997). In order to investigate ROS-triggered cell responses, we employed butyl hydroperoxide.

Treatment of *Nicotiana tabacum* (cv. Xanthi) cell suspension cultures with butyl hydroperoxide (1 mM) induced an

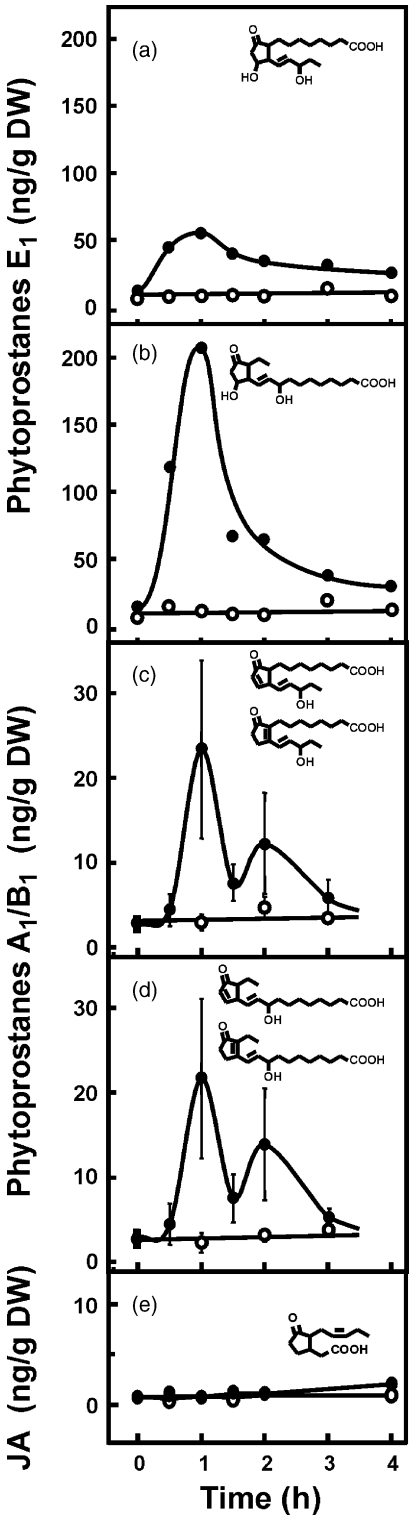

Figure 4. Time course of endogenous phytoprostane and JA levels in tobacco cell cultures when treated with 1 mM butyl hydroperoxide (closed circles) or water (open circles).
PPE_1 type I (a), PPE_1 type II (b), PPA_1/B_1 type I (c), PPA_1/B_1 type II (d), and JA (e) levels (ng g^{-1} of DW) were determined by GC-NCI-MS. Each data point is the average of measurements from two independent experiments (a,b,e) or the mean (±SE) of measurements from three independent experiments (c,d).

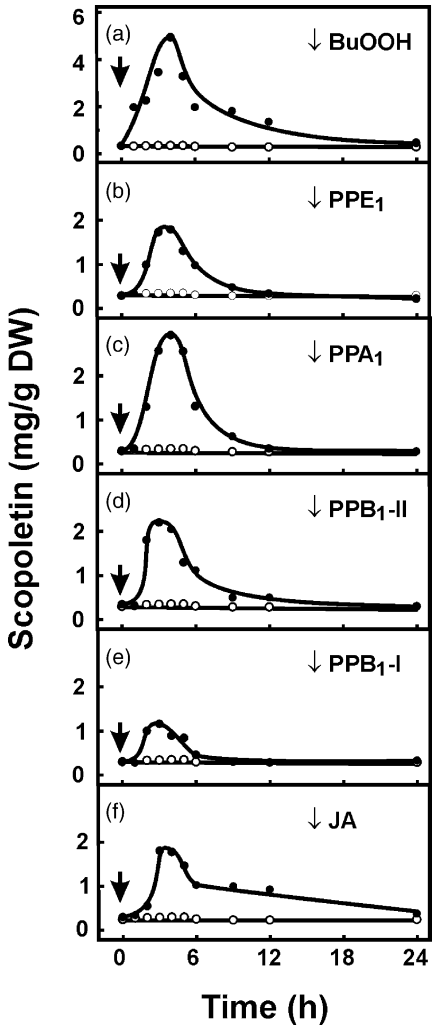

Figure 5. Time course of endogenous scopoletin levels in tobacco cell cultures when treated with butyl hydroperoxide (closed circles), different oxylipins (closed circles), or water (open circles).
Scopoletin levels in the cell culture medium were determined by HPLC after addition of 1 mM butyl hydroperoxide (a), 10 μM PPE_1 (b), 10 μM PPA_1 (c), 10 μM PPB_1-II (d), 10 μM PPB_1-I (e), or 10 μM JA (f). Concentrations are expressed as milligram of scopoletin per gram of cells (DW). Each data point is the average of measurements from two independent experiments.

accumulation of a characteristic tobacco phytoalexin, scopoletin, in the medium of tobacco cell cultures. A more than 16-fold increase in scopoletin levels (Figure 5a) was observed after the addition of 1 mM butyl hydroperoxide to the suspension culture. Maximum levels of scopoletin (4.9 mg g^{-1}) in the medium, calculated per gram of DW, were observed after 4 h. Thereafter, scopoletin levels almost decreased to baseline levels (0.3 mg g^{-1} of DW).

Cyclopentanone and cyclopentenone fatty acids of the jasmonate type induce phytoalexins in many, if not all, plant species (Gundlach and Zenk, 1998; Gundlach *et al.*, 1992). In tobacco, for instance, methyl jasmonate (MeJA)

induces the biosynthesis and accumulation of scopoletin in the extracellular medium of tobacco cell cultures (Sharan *et al.*, 1998). In fact, the time course of scopoletin accumulation in the medium of tobacco cell cultures was similar after addition of butyl hydroperoxide (1 mM) or jasmonic acid (10 μM) (Figure 5a,f). However, jasmonic acid was not induced by butyl hydroperoxide clearly (Figure 4e) and could not be responsible for the observed scopoletin accumulation under peroxide stress.

Phytoprostanes are potent inducers of scopoletin in tobacco cell cultures

The biochemical mechanisms by which ROS mediate several cellular events are not known. However, one inevitable consequence of enhanced ROS formation is the formation of an array of lipid peroxidation products, including isomeric prostaglandins, the isoprostanes, in animals (Lawson *et al.*, 1999) or structural congeners of jasmonates, the phytoprostanes, in plants. In order to explore if phytoprostanes are innocent by-products/markers of lipid peroxidation or biologically active oxylipins, different phytoprostanes were added to tobacco cell cultures and scopoletin levels were monitored.

Application of a mixture of E_1-phytoprostanes obtained from *in vitro* auto-oxidation of linolenate (comprising theoretically of 32 different isomers) at a total concentration of 10 μM to tobacco cell cultures led to an almost sixfold induction of scopoletin in the culture medium after 4 h (Figure 5b). However, because of the complexity of the isomer mixture (32 isomers), the mixture still contained yet unidentified linolenate peroxidation products. Thus, it could not be excluded that minor oxidized linolenate species present in the isomer mixture could elicit scopoletin formation. In contrast, partial synthesis of PPA_1 (16 isomers), PPB_1 type I (two isomers) and PPB_1 type II (two isomers) yielded chemically pure phytoprostanes as judged by HPLC, GC–MS, and NMR. As shown in Figure 5(c,d,e), all cyclopentenone phytoprostanes induced scopoletin accumulation with a similar time course as seen with butyl hydroperoxide (Figure 5a), JA (Figure 5f) or PPE_1 (Figure 5b). In direct comparison with JA (sixfold induction), scopoletin induction after 4 h was stronger with PPA_1 (10-fold) and PPB_1-II (sevenfold) and less with PPB_1-I (twofold). When PPA_1 and PPB_1 were added at a concentration of 50 μM to tobacco cell cultures, no increase in JA levels was observed, indicating that phytoprostanes do not mediate their effects via jasmonic acid biosynthesis (data not shown), which also is apparently not biosynthesized in response to butyl hydroperoxide in tobacco cell cultures (Figure 4e).

Scopoletin is known to accumulate in solanaceous plants upon infection and is generally considered to be an antimicrobial (Goy *et al.*, 1993) and antiviral (Chong *et al.*, 2002) phytoalexin. In addition, it has been shown that scopoletin acts as a scavenger of hydrogen peroxide in the presence of peroxidase not only *in vitro* but also *in vivo* (Chong *et al.*, 2002). However, other plant species biosynthesize phytoalexins that belong to other classes of natural products.

Elicitation of phytoalexin accumulation by phytoprostanes is not limited to *Nicotiana tabacum* (Solanaceae). PPA_1 and PPB_1 (at a concentration of 50 μM tested) also induced a dramatic accumulation of benzophenanthridine alkaloids in cell cultures of *Eschscholzia californica* (Papaveraceae), as well as of flavonoids in cell cultures of *Crotalaria cobalticola* (Fabaceae). Thus, biologic activities of phytoprostanes are apparently not limited to certain species and plant families (data not shown). Results obtained so far suggest that phytoprostanes are capable of inducing processes that are relevant for plant defense against microorganisms.

Induction of jasmonic acid and E_1-, A_1- and B_1-phytoprostanes in tomato infected with Botrytis cinerea

Having established that phytoprostanes can be generated abundantly during oxidative stress imposed by exogenous peroxides, it was of interest to investigate whether these oxylipins are also induced *in vivo*. In mammals, it has been well established that the levels of isoprostanes are inevitably elevated in a variety of diseases associated with enhanced formation of ROS, and thus isoprostanes are widely used as markers of oxidative stress in animals and humans *in vivo* (Lawson *et al.*, 1999; Pratico *et al.*, 2001). However, the production of ROS through an oxidative burst is a hallmark not only of certain mammalian but also of many plant defense responses, i.e. during plant–pathogen interactions (Bolwell, 1999). A large percentage of plant pathogens are biotrophs that require compounds from living host cells. Recognition of a pathogen attack triggers a hypersensitive reaction (HR) in the plant, which includes generation of ROS and local cell death. HR is considered to be one of the most important factors in impeding growth of biotrophic pathogens. *B. cinerea* is a necrotrophic fungal pathogen that attacks over 200 different plant species including tomato. In contrast to most biotrophs, *B. cinerea* is not deterred by cell death but rather utilizes the plant HR response to kill plant cells on which *B. cinerea* can feed on (Govrin and Levine, 2000). Infection of several plant species with *B. cinerea* leads to an increase in the levels of superoxide anions, hydrogen peroxide, end products of lipid peroxidation such as aldehydes, and stable free radical Fe(III) signals in electron paramagnetic resonance spectra in the apparently healthy tissue adjacent to the soft-rotted areas (Govrin and Levine, 2000; Muckenschnabel *et al.*, 2001). If isoprostane levels reflect oxidative stress not only in animals but also in plants, an increase in phytoprostane levels in infected

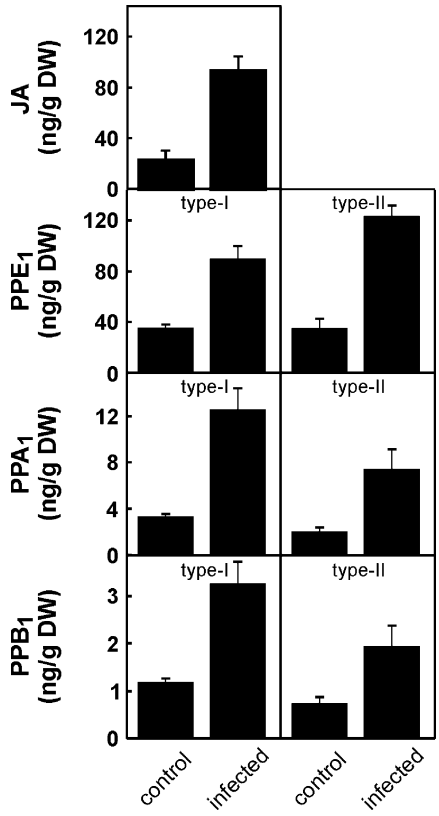

Figure 6. Levels of JA and phytoprostanes in tomato (*Lycopersicon esculentum* cv. Moneymaker) infected with *Botrytis cinerea*.
Levels of JA and phytoprostanes were determined by GC-NCI-MS in leaves of control plants and *B. cinerea*-infected plants 48 h after infection.

plants is to be expected to occur as a consequence of ROS formation.

Leaves of tomato plants were infected with a *B. cinerea* spore suspension. After 48 h of growth under humid conditions, spreading lesions developed (7% of the total leaf area). Leaves were collected, and oxylipin levels were determined in three independent experiments. Uninfected leaves (controls) were taken from the same plants next to the infected leaves. Levels of JA, PPE_1, PPA_1, and PPB_1 in control leaves were 22, 70, 5, and 2 ng g^{-1} of DW, respectively (Figure 6). In infected leaves, there was a three- to fourfold increase in the levels of all cyclic fatty acids. Thus, the phytoprostane pathway can be triggered independently of the JA pathway (Figure 4) or simultaneously (Figure 6), depending on the environmental conditions.

Activation of mitogen-activated protein kinases by cyclopentenone A_1- and B_1-phytoprostanes

Despite the recognition of ROS as central actors in stress and wound responses, pathogen defense, and regulation of cell cycle and cell death, little is known about how the ROS signal is perceived and transduced in plant cells. Activation of MAPK is a common reaction of plant cells in defense-related signal transduction pathways. Recently, it has been reported that hydrogen peroxide is a potent activator of MAPK in *Arabidopsis* leaf cells (Kovtun *et al.*, 2000). The effect of cyclopentenone phytoprostanes on MAPK activation was tested in tomato cell cultures treated with a solution of PPA_1 (75 μM), PPB_1 type I (75 μM) or type II (75 μM) for 5 min. Tyrosin-phosphorylated proteins were immunoprecipitated with a phospho-Tyr-specific MAPK antibody and analyzed by an in-gel kinase assay with the model substrate, myelin basic protein (MBP). As shown in Figure 7(a), both PPA_1 types I/II and PPB_1-II resulted in fast and strong activation of an MBP phosphorylating kinase activity, and thus a putative MAPK. In contrast, neither MeJA nor PPB_1-I resulted in activation of a kinase activity. Results indicate that MeJA and different classes/isomers of phytoprostanes exert structure-specific effects, and thus their biologic properties are not simply because of their physiochemical properties.

Cyclopentenone A_1- and B_1-phytoprostanes differentially induce extracellular invertase (Lin6) and phenylalanine ammonia lyase (PAL) in tomato

Oxidative stress, pathogen infection, wounding, and other biotic and abiotic stresses trigger gene induction of different subsets of genes involved in primary and secondary

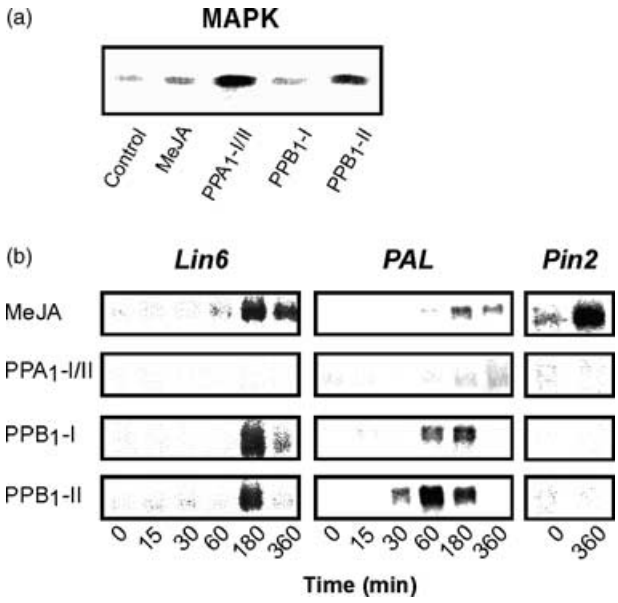

Figure 7. Activation of MAP kinase and induction of mRNA for extracellular invertase (*Lin6*), phenylalanine ammonia lyase (*PAL*), and proteinase inhibitor 2 (*Pin2*) by phytoprostanes in tomato cell cultures.
(a) Tomato cell suspension cultures were treated with 75 μM of MeJA, PPA_1 types I and II, PPB_1 type I, PPB_1 type II or water (control). Samples were taken after 5 min and analyzed by in-gel kinase assay.
(b) Total RNA was isolated from tomato cell suspension cultures treated with 75 μM of MeJA, PPA_1 type I, PPA_1 type II, PPB_1 type I, or PPB_1 type II. RNA was used for RNA gel blots hybridized with probes for *PAL*, *Lin6*, and *Pin2*.

metabolism. In order to study the effect of phytoprostanes on gene induction, we selected two genes that are commonly upregulated by a variety of stresses. Extracellular invertase from tomato (*Lin6*) was chosen as a gene involved in primary metabolism. Upregulation of extracellular invertase in response to stress-related stimuli increases the supply of carbohydrates locally and thus provides additional metabolic energy for activation of a cascade of defense reactions (Roitsch, 1999). *PAL* is another gene commonly upregulated by different stresses, which is involved in secondary metabolism. PAL may provide cells with cinnamic acid, a central precursor for lignin, and a variety of phytoalexins. As shown in Figure 7(b), stimulation of tomato cell suspension cultures with 75 μM MeJA (positive control), PPB_1 type I or II led to an increase in *Lin6* mRNA levels after 3 h. In contrast, PPA_1 did not induce *Lin6*. MeJA, PPB_1 types I and II also induced *PAL* expression, however, with a different time course. PPB_1 types I and II transiently increased PAL mRNA levels as early as 30–60 min. *PAL* induction by MeJA occurred later and was consistently lower than that with PPB_1. Interestingly, PPA_1, which strongly induced the PAL-dependent metabolite scopoletin in tobacco cells, did not induce expression of PAL in tomato. A marker gene for MeJA, *Pin2*, was also assayed. As expected, *Pin2* was strongly induced by MeJA. However, none of the cyclopentenone phytoprostanes did induce *Pin2* expression, indicating differential induction of defense genes by cyclic fatty acids (Figure 7b). Results suggest that phytoprostanes have their own spectrum of biologic activities, which partially overlaps with the spectrum of biologic activities of other cyclic oxylipins, such as 12-OPDA and MeJA/JA.

Glutathione-S-transferase1 is induced in Arabidopsis thaliana *by A_1- and B_1-phytoprostanes*

Previously, it has been shown that the cyclopentenone 12-oxo-phytodienoic acid (12-OPDA), but not JA, induces glutathione-S-transferase1 (*GST1*) expression in *Arabidopsis* (Stintzi *et al*. 2001). Cyclopentenone phytoprostanes, PPA_1 and PPB_1, share a structural element with 12-OPDA that has been identified as a key feature of a variety of compounds that induce *GST1* gene expression in *Arabidopsis* (Vollenweider *et al*. 2000), namely a chemically reactive α,β-unsaturated carbonyl group. In order to evaluate the potency of cyclopentenone phytoprostanes with respect to *GST1* induction, we infiltrated PPA_1, PPB_1-I and PPB_1-II into leaves of a transgenic *Arabidopsis* line expressing a *GST1* promoter:*GUS* (β-glucuronidase) reporter gene construct. Water (control) or test compounds (4 nmol $leaf^{-1}$) were infiltrated through the stomata as described (Vollenweider *et al*., 2000), and GUS activity was measured after 3, 6, and 24 h. After 24 h, GUS activity was dramatically increased by PPA_1, PPB_1-I, or PPB_1-II (induction of 11-, 11-, or 14-fold over control leaves, respectively), indicating strong induction of the *GST1* promoter in *Arabidopsis* (Figure 8). Thus, cyclopentenone phytoprostanes PPA_1 and PPB_1 may trigger an essential component of the plant electrophile detoxification system, which covalently inactivates electrophiles that would otherwise damage cellular proteins. When administered exogenously, PPA_1 and PPB_1 are rapidly taken up by tobacco cells and metabolized within a few hours to yet unknown metabolites (data not shown). In animals, cyclopentenone isoprostanes have been shown to be rapidly inactivated by conjugation to glutathione, a reaction catalyzed by GST (Chen *et al*., 1999). Interestingly, a great variety of electrophiles that can potentially induce GSTs are produced in oxidatively damaged cells. The growing list of these compounds (Vollenweider *et al*., 2000) includes ketodienes, hydroxynonenal, malondialdehyde, and various other unsaturated aldehydes and cyclopentenone phytoprostanes.

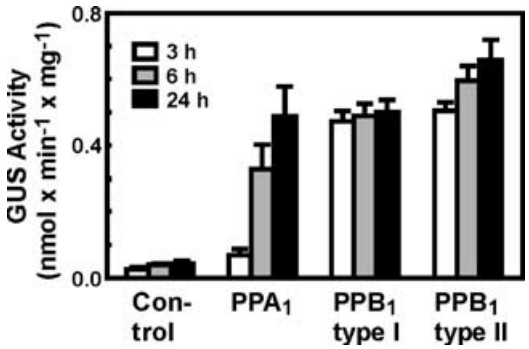

Figure 8. Activation of the *GST1* promoter in transgenic *Arabidopsis thaliana* (Col-0) leaves containing the β-glucuronidase (GUS) coding region driven by the *GST1* promoter after infiltration of cyclopentenone phytoprostanes.
Water (control) or phytoprostanes (4 nmol $leaf^{-1}$) were infiltrated through the stomata, and GUS activity was measured after 3, 6, and 24 h. Values are the mean (±SD) of three independent experiments.

Discussion

Free-radical-catalyzed cyclization of α-linolenic acid leads to highly unstable endoperoxide phytoprostanes (PPG_1), which decompose rapidly in aqueous environment. PPG_1 can be reduced to PPF_1 or rearranged to PPE_1 and PPD_1. We have previously shown that the phytoprostane pathway is apparently present in all plant species because of the fact that the only requirements for phytoprostane formation (linolenic acid, molecular oxygen, and ROS) occur ubiquitously in plants (Imbusch and Mueller, 2000a; Mueller, 1998; Parchmann and Mueller, 1998). A_1- and B_1-phytoprostanes are no exceptions to this rule. In the absence of appropriate amounts of radical scavengers, free radicals initiate rapid phytoprostane formation not only *in vitro* but also *in vivo*. Once initiated, phytoprostane formation is a

self-propagating process, which can only be aborted by radical chain breakers or oxygen/fatty acid deprivation *in vitro* as well as *in vivo*. Thus, the formation of isoprostanes reflects oxidative stress *in vivo* and the relative incapacity of the antioxidative mechanisms of living cells to suppress free radicals. Notably, isoprostanes in animals have been proven to represent reliable markers of oxidative stress *in vivo* (Jackson Roberts and Morrow, 2000; Pratico *et al*., 2001; Roberts and Morrow, 2002).

In plants, peroxides or ROS generated during a plant–pathogen interaction inevitably shift the cellular redox balance to the pro-oxidative side, and hence lead to enhanced formation of phytoprostanes (Figures 4 and 6). Surprisingly, phytoprostane accumulation initiated by exogenous peroxides (Figure 4) or copper ions (Imbusch and Mueller, 2000b) in plant cell cultures is a transient process. Although all phytoprostanes shown in Figure 1 can be generated non-enzymatically *in vitro*, it is possible that enzymes participate in certain steps of the phytoprostane pathway *in vivo*. It has been shown that isoprostanes in animals (Chen *et al*., 1999), and likely in plants also (Imbusch and Mueller, 2000a), are formed in membrane lipids *in situ* where the bulk of cellular fatty acids and isoprostanes is found esterified in lipids. Isoprostanes and other oxidized lipids in membranes are detrimental to membrane function/cellular integrity and can be cleaved by lipases from glycerolipids. Thereby, membrane repair is initiated and free isoprostanes are released (Morrow *et al*., 1992). Thus, yet to be identified lipases may play a key role in the liberation of pre-formed phytoprostanes from membranes.

Conversion of linolenate (free or esterified) into PPG_1 is almost certainly a non-enzymatic, free-radical-catalyzed process that yields both regioisomers in a 1 : 1 ratio. Rearrangement of PPG_1 (half-life $<$ 5 min) to PPE_1 in an aqueous environment occurs rapidly and also, most likely, does not require enzyme catalysis. However, it has not been established yet whether or not enzymes are involved in the further downstream reactions that occur more slowly *in vitro* (i.e. reduction of the side-chain hydroperoxide groups or dehydration/isomerization reactions). Although E_1-, A_1-, and B_1-phytoprostanes are chemically relatively stable compounds in the physiologic pH range, their observed half-life *in vivo* appears to be less than 30 min, suggesting that rapid metabolism of these compounds takes place at least in tobacco cell cultures (Figure 4). Hence, phytoprostane levels most likely can be regulated on the one hand by the rate of their formation/release from membranes and on the other by the metabolic capacity of plant cells.

Data obtained so far indicate that at least two pathways co-exist in plants that lead to cyclic fatty acids (Figure 9). Surprisingly, the cyclopentane and cyclopentenone products of the non-enzymatic phytoprostane pathway occur in the same concentration range as the products of the jasmonate pathway in healthy, untreated plants. Both types of oxylipins can be induced *in vivo*. JA synthesis can be triggered specifically by the interaction of extracellular ligands with membrane receptors, while phytoprostane formation can be triggered by ROS. Both processes may coincide, i.e. in plant–pathogen interactions (Figure 6), or be activated separately (Figure 4).

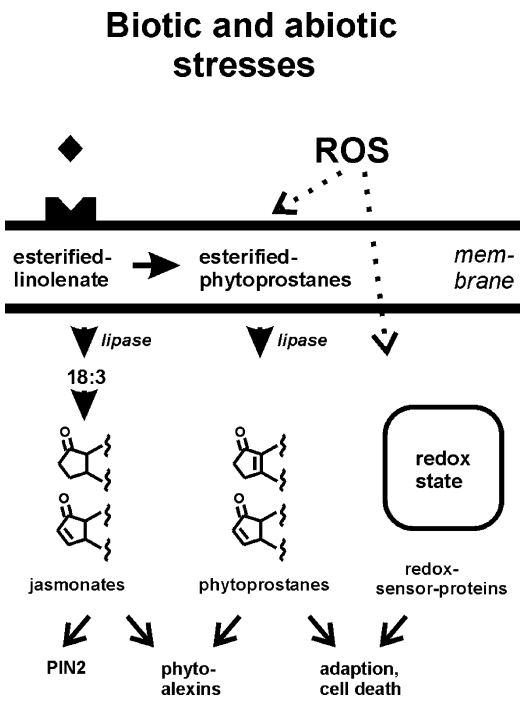

Figure 9. Stress-activated oxylipin signaling pathways in plants.
In plants, two major pathways leading to cyclic oxylipins start from esterified linolenate in membrane lipids. The lipoxygenase/allene oxide synthase pathway is triggered by specific ligands (such as systemin) that activate a lipase through membrane receptors. Free linolenate (18 : 3) is converted by a constitutive cascade of JA-biosynthesizing enzymes to jasmonate signals. A second oxylipin pathway is triggered by reactive oxygen species (ROS), which initiate oxidation of linolenate in membrane lipids yielding esterified phytoprostanes, which can be released by a lipase. We propose that free phytoprostanes act as mediators of oxidative stress. In addition, ROS may alter the cellular redox status directly and modulate gene expression in concert with octadecanoid signals (Bolwell, 1999).

Are phytoprostanes nothing more than by-products or markers of lipid peroxidation? To this end, our results infer that exogenously administered phytoprostanes have a serious impact on cell signaling (rapid activation of MAPK) and several cellular functions encompassing primary metabolism (induction of extracellular invertase), secondary metabolism (induction of PAL and secondary metabolites), and finally detoxification (induction of GST). As phytoprostanes are not only formed endogenously in the same concentration range as that of jasmonates but also display biologic activities at similar concentrations, the results suggest that phytoprostanes are mediators of oxidative stress *in vivo*.

Oxidative stress has been shown to induce adaptive responses that limit the consequences of oxidative injury.

However, the biochemical processes involved in the adaptive responses are not well understood. As biologically active phytoprostanes rapidly accumulate in oxidatively damaged cells, they may be parts of a signal transduction system that triggers certain adaptive reactions. For instance, phytoprostane-inducible phytoalexins may protect damaged tissues from invading microorganisms. Moreover, a variety of lipid peroxidation products including cyclopentenones are reactive electrophiles that can modify proteins covalently (Vranova *et al.*, 2002). Hence, GST (induced by cyclopentenone phytoprostanes in concert with a variety of lipid electrophiles) catalyzes conjugation of electrophiles to glutathione, and thus can prevent excessive protein damage. Finally, enhanced glucose supply provided by extracellular invertase can promote tissue regeneration.

Yet, the exact role and function of phytoprostanes in plant physiology remains to be elucidated in detail. We propose a model (Figure 9) in which phytoprostanes are components of an oxidant injury sensing, archaic signaling system that serves to protect plants from various stresses associated with increased free radical production. Our findings provide a rational basis to explore a novel concept in which plant isoprostanes act as mediators of oxidative stress in plants as has been proposed for isoprostanes in animals.

Experimental procedures

Chemicals and materials

Methyl jasmonate was obtained as a racemic mixture from Serva (Heidelberg, Germany), and JA was prepared by alkaline hydrolysis of the methyl ester. Oxygen-18 gas (99.1 at.% ^{18}O) was obtained from Isotec (Miamisburg, OH, USA). Prostaglandins and 12-OPDA were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Silica and aminopropyl solid phase extraction glass columns (500 mg) and thin layer chromatography (TLC) plates (Polygram SIL G/UV_{254}) were obtained from Macherey and Nagel (Düren, Germany).

Cell cultures and elicitor treatment

Cell suspension cultures were obtained from the departmental culture collection and grown as described by Gundlach *et al.* (1992). Cells were harvested under sterile conditions by suction filtration, re-suspended in 1 l flasks containing 250 ml of the medium or 300 ml flasks containing 80 ml of medium, and used after a growth period of 3 days directly for various experiments.

For scopoletin analysis, 9 g of *Nicotiana tabacum* cv. Xanthi cells were grown in 100 ml of LS medium for 3 days. Lipids (dissolved in 100 μl of methanol) were added to yield a final concentration of 10 μM. Methanol (100 μl) was added to the control cells. Samples (5 ml) were taken at the time points indicated and centrifuged (2000 ***g*** for 10 min), and the supernatant was directly subjected to HPLC analysis as described (Keinänen *et al.*, 2001; Sharan *et al.*, 1998). For each time point, additional samples were taken for the determination of FW and DW of the cells.

Preparation of phytoprostanes A_1 and B_1

Oxygen-18-labeled and unlabeled PPE_1 were purified from linolenate auto-oxidation mixtures and converted to PPB_1 by base-catalyzed dehydration as described by Parchmann and Mueller (1998). PPA_1 were prepared by acid-catalyzed isomerization of PPE_1: PPE_1 (5 mg) were dehydrated to PPA_1 with a mixture of water:acetic acid:phosphoric acid (10 : 3 : 2, v/v) at room temperature for 12 h. PPA_1 were extracted with diethyl ether, taken to dryness, reconstituted in chloroform, and applied to a silica SPE column (500 mg). The column was washed with 3 ml of chloroform, and PPA_1 were eluted with 6 ml of diethyl ether containing 2% acetic acid. Separation of PPA_1 regioisomers was performed by HPLC on a Lichrospher 100 RP 18ec column (5 μm particle size, 250 mm × 8 mm; Merck, Darmstadt, Germany). PPA_1 were eluted with a mixture of acetonitrile:methanol:water:acetic acid (19 : 22 : 59 : 0.1, v/v) at a flow rate of 3.5 ml min^{-1}. The regioisomers were detected at a wavelength of 217 nm and collected. The PPA_1 preparation was essentially free of PPB_1 isomers as judged by HPLC. For quantification of PPA_1, samples were spiked with 5 μg of prostaglandin A_1 as internal standard and were analyzed by HPLC.

To confirm the structures of PPA_1, they were analyzed by GC–MS as their corresponding methyl ester, trimethylsilyl ether derivatives. PPA_1 isomerized almost quantitatively to the thermodynamically more stable PPB_1 in the injector port of the GC (more than 97%). However, the remaining PPA_1 derivatives could be separated from PPB_1 by GC and measured by MS in the electron impact (EI) mode (Figure 2c,d). Methoximation prevents isomerization of the A-ring system, and therefore PPA_1 were also measured as their corresponding methyl ester, methoxime, trimethylsilyl ether derivatives (Figure 2a,b). Two regioisomeric PPA_1 exist (types I and II), each of which theoretically comprises of eight isomers. The structure of each of the two regioisomeric PPA_1 was unequivocally established by NMR after base-catalyzed conversion of PPA_1 regioisomers to their corresponding PPB_1 isomers.

NMR analysis of PPA_1 as PPB_1 derivatives

Each of the two PPB_1 regioisomers obtained from PPA_1 regioisomers comprises of only one racemic isomer. Structures of phytoprostanes B_1 types I and II were established by NMR on a Bruker AMX 600 instrument (Bruker, Rheinstetten, Germany). The solvent peak was used as internal reference ($CDCl_3$: δH 7.24, δC 77.0).

^1H NMR ($CDCl_3$) of PPB_1 type I (p.p.m.): δ 0.99 (t, H18abc, $J = 7.3$ Hz), 1.31 and 1.39 (m, H4-H7, 8 protons), 1.63 (m, H3ab), 1.67 (ddm, H17, $J = 11.5/7.3$ Hz), 2.26 (ddd, H8ab, $J = 8.9/6.0/2.6$ Hz), 2.34 (t, H2ab, $J = 7.2$ Hz), 2.42 (m, H11ab), 2.64 (m, H12ab), 4.31 (ddd, H16, $J = 11.5/5.8/1.1$ Hz), 6.25 (dd, H15, $J = 15.8/5.8$ Hz), 6.81 (d, H14, $J = 15.8$ Hz).

For PPB_1 type II: δ 1.01 (t, H18abc, $J = 7.6$ Hz), 1.35 and 1.45 (m, H4-H7, 8 protons), 1.62 (m, H8ab), 1.64 (m, H3ab), 2.30 (q, H17ab, $J = 7.6$ Hz), 2.35 (t, H2ab, $J = 7.5$ Hz), 2.42 (m, H14ab), 2.64 (m, H13ab), 4.33 (dd, H9, $J = 12.2/6.1$ Hz), 6.24 (dd, H10, $J = 15.8/6.0$ Hz), 6.80 (d, H11, $J = 15.8$ Hz).

^{13}C NMR ($CDCl_3$) of PPB_1 type I (p.p.m.): δ 9.61 (C18), 22.74 (C8), 24.54 (C3), 25.62 (C12), 28.28, 28.32, 28.40, 28.87 (C4–C7), 30.13 (C17), 33.44 (C2), 33.82 (C11), 73.67 (C16), 124.13 (C14), 139.50 (C15), 141.54 (C9), 162.98 (C13), 176.41 (C1), 209.58 (C10).

^{13}C NMR ($CDCl_3$) of PPB_1 type II (p.p.m.): δ 13.49 (C18), 16.32 (C17), 24.62 (C3), 25.24, 28.90, 29.80, 29.23 (C4–C7), 25.60 (C13), 33.34 (C2), 33.86 (C14), 37.22 (C8), 72.44 (C9), 123.87 (C11), 139.93 (C10), 142.87 (C16), 162.52 (C12), 176.30 (C1), 209.43 (C15).

Sample preparation and GC–MS analysis of phytoprostanes A_1 and B_1 from plant material

For PPA_1/B_1 analysis, plant material (5–8 g of FW) was suspended in 20 ml of cold brine containing 0.05% of 2,6-di-*tert*-butyl-4-methylphenol (w/v), 20 mg of triphenylphosphine, and 0.2 ml of 1 M citric acid. $[^{18}O]PPB_1$ (20 ng) was added as the internal standard. After addition of 20 ml of diethyl ether, the mixture was homogenized for 3 min with a high-performance disperser (Ultraturrax T 25, IKA-Werk, Germany) at 24 000 r.p.m. and then centrifuged for 10 min at 2000 ***g***. The ether phase was removed, taken to dryness under a stream of nitrogen, and re-constituted in diethyl ether. Samples were applied to an aminopropyl SPE column (500 mg). The column was washed with 3 ml of chloroform: 2-propanole (80 : 20, v/v), and PPA_1/B_1 were eluted with 6 ml of diethyl ether containing 2% acetic acid. PPE_1 and PPF_1 were retained on the column. The extracts were taken to dryness and dissolved in chloroform. Samples were applied to a silica SPE column (500 mg), and the column was washed with 3 ml of hexane:diethyl ether:acetic acid (67 : 33 : 1, v/v). PPA_1/B_1 were eluted with 6 ml of diethyl ether containing 2% acetic acid. The extracts were taken to dryness and dissolved in 200 μl chloroform.

For NCI-GC–MS analysis, PPA_1/PPB_1 were derivatized with 10 μl of pentafluorobenzyl bromide and 10 μl of *N*,*N*-diethylisopropylamine at 40°C for 45 min. The mixture was taken to dryness, and trimethylsilyl ether derivatives were prepared with 20 μl of bis(-trimethylsilyl)trifluoroacetamide at 40°C. The mixture was dissolved in hexane and applied to a silica SPE column (500 mg). PPA_1/B_1 were subsequently eluted with 6 ml of hexane:diethyl ether (67 : 33, v/v) and taken to dryness. For GC–MS analysis, the sample was dissolved in 20 μl of hexane, and 2 μl was analyzed. As PPA_1 are almost quantitatively isomerized to PPB_1 in the injector of the GC, PPA_1 and PPB_1 were collectively quantified as PPB_1 against the internal standard $[^{18}O]PPB_1$ (Figure 3a). A number of control experiments were performed to validate the analytical methods. Blank samples were spiked with $[^{18}O]PPB_1$ (500 ng) and worked up as described above. GC–MS measurements of the $[^{18}O]PPB_1$ derivatives revealed that unlabeled PPB_1 could not be detected within the limit of detection, indicating that exchange of the oxygen-18 label does not take place during sample preparation or GC–MS measurements. Additional control experiments were performed to exclude the possibility that E-ring compounds are converted to A_1/B_1-ring compounds during sample preparation. Blank samples were spiked with 2 μg of PPE_1 and 50 ng of $[^{18}O]PGB_1$ (internal standard). Samples were purified as described above. While $[^{18}O]PGB_1$ was clearly detectable, degradation products of PPE_1, PPA_1 and PPB_1, could not be detected by GC–MS. In additional experiments, the recovery of PPA_1 and PPB_1 (relative to the internal standard) was checked. Blank samples were either spiked with PPA_1 (50 ng) and $[^{18}O]PPB_1$ (50 ng) or PPB_1 (50 ng) and $[^{18}O]PPB_1$ (50 ng), worked up as described above and analyzed by GC–MS. Peak areas of unlabeled cyclopentenones were equivalent to the peak areas of the internal standard ($[^{18}O]PPB_1$) within experimental error (± 5%, $n = 3$), indicating that the analytical method could be used for quantification of PPA_1/B_1.

In order to determine the ratio of PPA_1 and PPB_1 in the sample, an aliquot of the plant tissue (20 g of FW) was extracted and purified through an aminopropyl column as described. The eluate of the aminopropyl SPE column was spotted on a TLC plate. PPB_1 was spotted on the plate (3 cm distance to the sample) and served as reference. The plate was developed in diethyl ether containing 2% of acetic acid. PPA_1 and PPB_1 co-migrated on the TLC plate. The region of the TLC plate corresponding to the PPB_1 standard was eluted with methanol. Prostaglandins A_1 and B_1 (300 ng) were added as reference compounds to check the efficiency of the following derivatization step. The eluate was dried under a stream of nitrogen. The residue was treated with 50 μl of a solution of methoxyamine HCl (25 mg) in dimethylformamide and incubated for 1 h at 40°C. Water (2 ml) and 2 ml of diethyl ether were added, and the solution was shaken. The organic phase containing the methoxime derivatives was dried under a stream of nitrogen and the pentafluorobenzyl ester, trimethylsilyl ether derivatives were prepared and analyzed as described above. Methoxime derivatives of PPA_1 and PPB_1 do not isomerize in the GC and could baseline separated by GC (Figure 3b). PPA_1 and PPB_1 were detected at *m/z* 408, and the ratio of the compounds was calculated from their corresponding peak areas. The concentrations of PPA_1 and PPB_1 could not be determined directly in the second experiment, as the required $[^{18}O]PPB_1$ standard would loose the label (located in the cyclopentenone ring) during methoximation. Therefore, the total amounts of PPA_1 and PPB_1 were calculated from the total concentration of PPA_1/B_1 (determined in the first experiment) and the ratio of PPA_1 to PPB_1 (determined in the second experiment).

Gas chromatography–mass spectrometry measurements

Measurements were performed on an Agilent 6890 gas chromatograph interfaced to a JEOL JMS-GC-Mate II-mass spectrometer. The MS source was set at 200°C and the electron energy at 200 eV. Methane was used as a reactant gas. PPA_1/B_1 were analyzed by NCI-GC–MS as previously described for PPB_1 (Parchmann and Mueller, 1998). JA analysis was performed as described by Mueller and Brodschelm (1994).

Infection of tomato leaves with Botrytis cinerea

Botrytis cinerea DC 3000 spores were grown on potato dextrose agar (Sigma, Deisenhofen, Germany) at room temperature. Spores were harvested by washing the culture plates with sterile malt extract (2% aqueous solution; Difco, Detroit, USA). Six needle-prick wounds were applied to the leaves of 5-week-old soil-grown *Lycopersicon esculentum* cv. Moneymaker plants, and these wounds were covered with 5 μl drops of a spore suspension (10^6 spores ml^{-1}). Controls were treated with 6 × 5 μl of the fungal growth medium (malt extract). For analysis of PPA_1 and PPB_1, 5 g of the plant material was harvested after 48 h.

Extraction of mRNA and RNA gel blot analysis

For the isolation of RNA, cells were harvested by centrifugation, snap-frozen in liquid nitrogen, and ground in the presence of liquid nitrogen. Total RNA was isolated as described (Chromczynski and Sacchi, 1987). Northern blot analysis was carried out as described previously (Godt and Roitsch, 1997).

In-gel kinase assay for MAPK

Cells were harvested by centrifugation, snap-frozen in liquid nitrogen, and ground in the presence of liquid nitrogen. The enzyme was extracted from the tissue, immunoprecipitated with a phospho-Tyr-specific monoclonal antibody 4G10 (UBI, Lake Placid, NY, USA), and analyzed by in-gel kinase assay with mylein basic protein (MBP, UPstate Biotechnology, Lake Placid, NY, USA) as substrate, as described previously (Link *et al.*, 2002; Zhang and Klessig, 1997). The activity was visualized by autoradiography and phosphor imager (Cyclone Phosphor Storage system, Madison, WI, USA).

GUS assay of transgenic Arabidopsis thaliana *plants transformed with a GST1 promoter-GUS fusion construct*

A transgenic *A. thaliana* cell line (Rate and Greenberg, 2001) containing the glutathione-S-transferase (*GST1*) promoter fused to β-glucuronidase (*GUS*) gene was obtained from J.T. Greenberg (Department of Molecular Genetics and Cell Biology, University of Chicago, USA). Phytoprostane solutions in water containing 0.5% methanol (4 nmol in 50 μl) were infiltrated through the stomata using a sterile syringe. Control leaves were treated the same way with water containing 0.5% methanol. For determination of GUS activity, leaves were collected and ground in 300 μl GUS extraction buffer (50 mM $NaPO_4$ (pH 7), 10 mM Na_2 EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, and 10 mM 2-mercaptoethanol). After centrifugation for 10 min (15 000 ***g***) at 4°C, 100 μl of the supernatant was mixed with 100 μl GUS assay solution (2 mM 4-methylumbelliferyl-D-glucuronide in extraction buffer). Fifty microliters were immediately removed and transferred to a stop solution (final concentration 0.3 M Na_2CO_3) to be used as the control. The rest of the mixture was incubated at 37°C for 1 h and stopped with 0.3 M Na_2CO_3. GUS activity was determined using a luminescence spectrometer (Perkin Elmer LS 30, Langen, Germany), and protein concentration of the tissue homogenate was determined with the Bradford reagent (Bradford, 1976) using bovine serum albumin as a standard.

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