Isoprostanes \( F_2 \) are arachidonate autoxidation products in mammals that have been shown to be induced during several human disorders associated with enhanced free-radical generation. Isoprostanes \( F_2 \) represent not only extremely reliable markers of oxidative stress in vivo, but they also exert potent biological effects. Therefore, it has been postulated that isoprostanes are mediators of oxidant injury in vivo. Higher plants, however, do not synthesize arachidonic acid or isoprostanes. Here we show that a series of isoprostane \( F_2 \) analogs termed phytoprostanes \( F_1 \) (previously dinor isoprostanes) are formed by an analogous pathway from \( \alpha \)-linolenate in plants. High-performance liquid chromatography and gas chromatography-mass spectrometry methods using \([^{18}O]3\) phytoprostanes \( F_1 \) as internal standard have been developed to quantify phytoprostanes \( F_1 \). In fresh peppermint (\textit{Mentha piperita}) leaves, phytoprostanes \( F_1 \) were found in free form (76 ng/g of dry weight) and at about 150-fold higher levels esterified in lipids. It is notable that these levels of phytoprostanes \( F_1 \) are more than two orders of magnitude higher than the basal levels of isoprostanes \( F_2 \) in mammalian tissues. Furthermore, wounding, as well as butyl hydroperoxide or cupric acetate stress triggered a dramatic increase of free and esterified phytoprostanes \( F_1 \). Thus phytoprostanes \( F_1 \) may represent a sensitive measure of oxidative damage in plants similar to isoprostanes in mammals. However, one of the most exciting issues to be clarified is the possibility that linolenate-derived phytoprostanes \( F_1 \) exert biological activities in plants and/or animals.

Free radical-catalyzed oxidation of arachidonate has been shown to lead to a complex isomeric mixture of prostaglandin-like compounds termed isoprostanes. Isoprostanes have attracted considerable attention since it has been shown that isoprostanes are formed in mammals at concentrations that exceed the levels of enzymatically formed prostaglandins by at least an order of magnitude (Morrow and Roberts, 1997). Furthermore, isoprostanes are dramatically induced by oxidative stress (Lawson et al., 1999) and have been shown to be biologically active in the low nanomolar range. Therefore, it has been postulated that isoprostanes represent mediators of oxidative cell injury (Rokach et al., 1997a). Since isoprostanes \( F_2 \) represent chemically stable end products of lipid peroxidation, they have found to be extremely reliable and sensitive markers of oxidative stress in vivo (Morrow et al., 1999).

Higher plants generally do not synthesize the precursor arachidonate required for isoprostane formation, but rather utilize \( \alpha \)-linolenic acid (the predominant polyunsaturated fatty acid in plants) for the formation of isoprostanoids and prostaglandin-like mediators of the jasmonate-type (Mueller, 1998). Linolenic acid (C18:3) can undergo free radical-catalyzed oxidation reactions similar to arachidonate (C20:4; Pryor et al., 1976; O’Connor et al., 1984) to yield C18 isoprostanoids (Fig. 1). Because the isoprostanoids derived from linolenate are two methylene groups shorter than those from C20 acids we have previously termed them dinor isoprostanes (Parchmann and Mueller, 1998; Mueller, 1998). However, C18 compounds derived from \( \beta \)-oxidation of isoprostanes (C20) in animals are also termed dinor isoprostanes, but differ from the plant dinor isoprostanes by the length of the two side chains (Chiabrando et al., 1999). To avoid confusion of terminology we suggest to term the plant prostanoids phytoprostanes.

We have recently elucidated the phytoprostane pathway in plants and have established that a group of prostaglandin/isoprostane E1- and F1-like compounds is formed in plant cells in vivo (Parchmann and Mueller, 1998; Imbusch and Mueller, 2000). The phytoprostane pathway theoretically yields several classes of phytoprostanes denoted with letters that specify the prostaglandin ring system according to the prostaglandin/isoprostane nomenclature (Fig. 1; Rokach et al., 1997b; Mueller, 1998). Each class represents a complex isomeric mixture that consists of two regioisomers (type I and type II). In addition, each regioisomer is theoretically comprised of 16 stereomers. In analogy to the mammalian isoprostane pathway, it is to be expected that phytoprostanes \( F_4 \) (PPF4) are among the most abundant isoprostanoids in plant tissues. Due to the isomeric complexity of PPF4, identification and quantitation of this class of compounds is technically difficult.

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Prostanoid analysis in plant tissues is further complicated by the occurrence of acyclic trihydroxylated fatty acid derivatives (Fig. 2) that have the same Mr as the cyclic trihydroxylated fatty acid isomers of the PPF1-type. Acyclic triols show similar chromatographic mobilities as prostanoids and may erroneously be identified as prostaglandins in various prostaglandin immuno- and bioassays (Hamberg and Gardner, 1992). Various acyclic triols may be formed via the peroxxygenase or epoxy alcohol synthase pathway in plants (Panossian et al., 1983; Hamberg and Hamberg, 1996; Hamberg, 1999). In addition, nonenzymatic formation of several acyclic triols by autoxidation (O'Connor et al., 1981) and photooxidation (Frankel, 1998) may not only take place in vitro, but also in vivo. Preparative and analytical methods were developed to specifically analyze and quantify PPF1 in vitro and in vivo. We show that PPF1 are major products of the isoprostane pathway in plants (Fig. 1). Furthermore, it is demonstrated that PPF1 accumulate dramatically in oxidatively stressed plant cell cultures and in wounded plants.

RESULTS

Preparation of PPE1 and PPF1

PPE1 and PPF1 were produced by autoxidation of α-linolenate in two different solvent systems. Bicyclic endoperoxides with the prostaglandin G-ring system are proposed to be common short-lived intermediates in the biosynthesis of phytoprostanes that readily rearrange to PPD1 and PPE1 in protic solvents (Fig. 1). Since endoperoxides are much less prone to spontaneous rearrangement in aprotic solvents, PPF1 may favorably be formed in aprotic solvents by reduction of endoperoxides (Fig. 1). To produce PPF1, linolenate (5 g) was dissolved in 50 mL of carbon tetrachloride and stirred under air for 10 d. In an alternate manner for optimized synthesis of PPE1, a mixture of methanol/water (1:1, v/v) was chosen as solvent. Phytoprostanes were quantified by gas chromatography-mass spectrometry (GC-MS) as described below. Yields of PPF1 were 2.3 mg/g of linolenate in the protic solvent and 11.7 mg/g in the aprotic solvent.
The protic solvent favored formation of PPE₁ (2.4-fold excess over PPF₁), whereas the aprotic solvent strongly favored PPF₁ formation (39-fold excess over PPE₁).

Purification, Identification, and Quantification of PPF₁

Autoxidation of linolenic acid as described above yields a complex mixture of peroxidized compounds. Endoperoxide and hydroperoxide groups of the reaction products were reduced to the corresponding hydroxy compounds by incubation of the autoxidation mixture with triphenylphosphine (1 g/g linolenic acid) for 30 min. A purification method was developed using tritiated prostaglandin F₂α (PGF₂α) as tracer to optimize and calculate the recovery of prostaglandin F-like compounds. The reduced linolenate autoxidation mixture (equivalent to 300 mg of fatty acid) was purified by silica solid phase extraction on columns containing 500 mg of silica (see “Materials and Methods” for details). The separation efficiency was checked by scintillation counting of the radioactivity in different fractions. Recovery of PGF₂α in the sample was 98% ± 1.6%. In addition, the absence of phytoprostanes in the washing fluids and the presence in the eluate was verified by thin layer chromatography (TLC) analysis using PPF₁ prepared by NaBH₄ reduction of authentic PPE₁ (Parchmann and Mueller, 1998) as reference compounds.

The mixture of PPF₁, theoretically comprised of 32 isomers, was analyzed by HPLC. PPF₁ were partially resolved on a C₁₈ reversed phase HPLC column (Fig. 3). GC-electron impact (EI)-MS analysis of the HPLC peaks after methylation and silylation of the samples showed that only peaks denoted with 1 to 4 eluted as single peaks from the GC column. PPF₁ regioisomers were identified by their EI spectra (Fig. 4, A and B). PPF₁ type I and type II reference spectra were recorded from PPF₁s obtained by NaBH₄ reduction of authentic PPE₁ type I and type II compounds (Parchmann and Mueller, 1998). HPLC peaks 1 and 3 contained PPF₁ type I isomers and HPLC peaks 2 and 4 contained type II isomers. HPLC peaks labeled with 5 to 7 (Fig. 3) contained mixtures of PPF₁ type I and II diastereomers.

High resolution MS revealed that PPF₁ regioisomers produce ions (Fig. 4, A and B) at m/z 217.1074 (C₉H₂₁Si₂O₂, trimethylsilyl [TMS]-O-CH=CH-CH=O-TMS) and 191.0928 (C₁₇H₅₃Si₃O₅, loss of C₂H₅). These ions are indicative for F-ring prostanoioids as they are generated by intramolecular rearrangement reactions of the cyclopentane F-ring system (Roberts et al., 1998). In addition, both regioisomers form ions at m/z 529.3207 (C₂₈H₅₃Si₃O₅, loss of C₆H₆), 468.3101 (C₂₅H₄₈Si₂O₄, loss of TMS-hydroxy [OH]), and 378.2578 (C₂₂H₃₈SiO₃, loss of 2 × TMS-OH). Fragment ions that are indicative for PPF₁ type I (Fig. 4A) were identified at m/z 439.2703 (C₂₃H₄₃Si₂O₄, loss...
of C₂H₅ and TMS-OH), 323.2041 (C₁₈H₃₁SiO, loss of C₂H₅, CH₂CH-OTMS, and TMS-OH), and 131.0889 (C₆H₁₅SiO, TMSO₁₅C₃H₆). Fragment ions indicative for PPF₁ type II compounds (Fig. 4B) are found at m/z 401.2342 [C₁₉H₄₁Si₃O₃, loss of (CH₂)₇COOCH₃], 311.1850 [C₁₆H₃₁Si₂O₂, loss of (CH₂)₇COOCH₃ and TMS-OH], and 195.1192 [C₁₁H₁₉SiO, loss of TMSO₁₅CH(CH₂)₇COOCH₃ and TMS-OH]. Different isomers of each regioisomer could, however, not be differentiated by MS.

The methyl ester TMS ether derivatives of PPF₁ type I and II were also analyzed by positive chemical ionization-MS (Fig. 4, C and D). The predominant ions produced from type I and II compounds are the molecular ion [M+H]⁺ at m/z 559 and three fragments at m/z 469, 379, and 289 derived from the sequential cleavage of TMS-OH from the three TMS ether groups of the molecules. The intensity of these ions varies greatly among different PPF₁ isomers. It is not possible to differentiate type I and II compounds by their chemical ionization spectrum.

PPF₁ were also analyzed as their corresponding pentafluorobenzyl (PFB)-ester, TMS derivatives in the negative ion chemical ionization (NICI) mode, which is an extremely sensitive detection method for prostanoids. NICI spectra of PPF₁ type I and II were identical, displaying only one major fragment ion at m/z 543 corresponding to the [M-PFB]²⁻ anion (Fig. 4, E and F).

Synthesis of Oxygen-18-Labeled PPF₁

GC-MS analysis requires an appropriate internal standard. In the case of PPF₁, which represents a mixture of 32 isomers, the use of a commercially available C₂₀ homolog such as PGF₁ would be straightforward, but unlikely to be reliable since various PPF₁ isomers and PGF₁ probably would have

![Figure 4. MS of PPF₁ type I and type II derivatives. PPF₁ were analyzed as their corresponding methyl ester, TMS ether derivatives in the EI (spectra A and B) as well as in the CI (spectra C and D) mode. PPF₁ were also analyzed as their PFB ester, TMS ether derivatives in the negative chemical ionization mode (spectra E and F). Type I compounds (A, C, and E) can only be differentiated from type II compounds (B, D, and F) by EI-MS. Characteristic cleavage points were indicated at the molecular formulae, see “Results” for details.](image-url)
different chromatographic properties and ionization efficiencies. The most appropriate retention time and quantification standard was found to be an isotopically labeled PPF$_1$ isomer mixture, which was prepared by oxidation of linolenate in an oxygen-18 atmosphere. GC-MS analysis of oxygen-18 labeled PPF$_1$ revealed that each isomer contained three $[^{18}\text{O}]$hydroxy groups as predicted by the mechanism of formation (Fig. 1) and that the GC peak pattern matched exactly that of the unlabeled PPF$_1$ isomer mixture (data not shown). An unlabeled PPF$_1$ standard solution of known concentration (see “Materials and Methods”) was used to quantify the mixture of $[^{18}\text{O}]_3$PPF$_1$ by GC-NICI-MS.

GC-NICI-MS of Phytosteranes in Plant Material

The most sensitive method for quantifying prostanoids by GC-MS described in the literature involves analyzing the PFB ester, TMS ether derivatives in the NICI mode. A drawback of the method is, however, that various enzymatically and nonenzymatically formed acyclic, trihydroxylated derivatives of linolenate cannot be differentiated from PPF$_1$s since they may behave similar on GC-MS and would yield ions of identical mass (O’Connor et al., 1984). However, acyclic oxygenation products of linolenic acid could not be detected by GC-EI-MS analysis of purified linolenate autoxidation mixtures (see above). In contrast, PPF$_1$s purified from plant sources with the sample preparation procedure used for purification of PPF$_1$s from linolenate autoxidation mixtures revealed a peak pattern on GC that largely matched the pattern of the internal standard, but showed additional non-PPF$_1$ derived peaks at m/z 543. A representative example (analysis of dried peppermint [Mentha piperita] leaves) is shown in Figure 5A. Because phytoprostanes F$_1$ contain only one double bond, whereas all acyclic triols contain two double bonds (Fig. 2), hydrogenation of the samples increases the molecular mass of the compounds by 2 and 4, respectively. Thus PPF$_1$ can be selectively quantitated by GC-NICI-MS even in the presence of acyclic triols. Levels of free PPF$_1$ in dried peppermint leaves were found to be 20.01 ± 0.76 µg/g of dry weight. After hydrogenation, the peak pattern of PPF$_1$ formed within the plant (at m/z 545) matched almost perfectly the peak pattern of synthetic $[^{18}\text{O}]_3$PPF$_1$ at m/z 551 (Fig. 5B). The major peaks in the chromatogram of the internal standard have all been shown to represent mixtures of PPF$_1$ regiosomers of type I and type II by co-injection of different purified PPF$_1$ isomers. The presence of a family of PPF$_1$ isomers in dried peppermint leaves, which apparently has the same isomer distribution as PPF$_1$ from autoxidized linolenate suggests that PPF$_1$ have been formed by autoxidation of endogenous linolenate.

Assay Parameters and Validation

The recovery of PPF$_1$ in different steps of the sample preparation method was estimated by spiking 300 mg of lime tree flowers (dry powder) with [5,6,8,9,11,12,14,15-3H]PGF$_2$ (specific activity of 179.00 Ci/mmol) prior to work-up. Recovery of radioactivity after various processing steps was determined by scintillation counting. Ether extraction (used for the measurement of free PPF$_1$) and modified Folch extraction/base hydrolysis (used for the determination of esterified plus free PPF$_1$), as well as the following silica solid phase extraction step were all performed with a recovery greater than 90%. After full sample preparation 60.57% ± 1.48% and 31.27% ± 8.97% of the...

Figure 5. GC-NICI-MS analysis of PPF$_1$ in dried peppermint leaves. PPF$_1$ were determined as PFB ester, TMS ether derivatives (A) and (B) prior catalytic hydrogenation. Ion chromatograms at m/z 543 and 545 display endogenous PPF$_1$ (20.01 ± 0.76 µg/g of dry weight), whereas m/z 549 and 551 display the triply oxygen-18 labeled internal standard. For quantification, peaks in the chromatograms of sample B were integrated together and the endogenous level of PPF$_1$ was calculated from the ratio of the peak areas at m/z 545 to that at m/z 551.
radioactivity were recovered in samples containing free and total PPF₁, respectively.

The quantification of endogenous PPF₁ was performed by using [¹⁸O]₃PPF₁ as an internal standard by comparison of the intensity of the peaks at m/z 545 co-eluting with the peaks of the internal standard at m/z 551. The lower limit of detection of PPF₁ (signal to noise ratio of 5:1) is in the range of 100 pg. Several procedures were performed to establish the accuracy of this assay. Precision was measured by analyzing aliquots of tobacco cells (170 mg of dry weight) obtained from a cell suspension culture 3 d after transfer to fresh medium. The mean of six replicate measurements of the ratio of m/z 545 to m/z 551 was found to be 1.07% ± 0.16% and corresponded to a concentration of 36.67 ± 5.77 ng/g of dry weight. Accuracy was assessed using aliquots of the same tissue. For this, 10 to 1,500 ng of unlabeled PPF₁ and 500 ng of [¹⁸O]₃PPF₁ were added to eight sample aliquots that were re-assayed in triplicate. A linear relationship between added and found PPF₁ was documented (r = 0.9890). The amount of endogenous PPF₁ measured in the precision experiment was subtracted from the total measured and the accuracy of the assay to measure the added 10 to 1,500 ng of PPF₁ was calculated. The accuracy of PPF₁ determinations varied with the level of PPF₁ to be measured (Fig. 6).

PPF₁ Levels Are Wound Inducible

To clarify if the large PPF₁ accumulation observed in harvested peppermint leaves (Fig. 5) is an early event triggered by wounding of living plants or occurs by autoxidation of lipids in dead plants we wounded fresh peppermint leaves with a nail stamp (12 nails/cm²). Time-dependent PPF₁ accumulation was measured over 6 h after wounding (Fig. 7, E and F). The concentration of free PPF₁s increased and reached a maximum level of 192 ng/g of dry weight (more than 4-fold over control levels) within 1 h and declined thereafter. A second increase was observed after 3 h. Levels of esterified PPF₁ rose after wounding, reached a maximum level of 4,000 ng/g of dry weight (more than 5-fold over control levels) after 1.5 h and decreased thereafter. The PPF₁ isomer distribution was found to be similar to that of the synthetic [¹⁸O]₃PPF₁ standard. Thus wounding appears to be accompanied by formation of free radicals, which trigger an appreciable synthesis of free and esterified PPF₁.

Although accumulation of PPF₁ in wounded plants does take place, levels of PPF₁ are well below the levels found in dried, stored plant powders, suggesting that massive PPF₁ formation takes place in dead plant material during storage.

Exogenous Addition of Butyl Hydroperoxide or Cupric Acetate Results in Dramatic Accumulation of PPF₁ in Plant Cells

To assess the effect of oxidative stress on PPF₁ levels in plant cells, California poppy (Eschscholzia californica) cell suspension cultures were treated with 10 mM t-butyl hydroperoxide. A dramatic increase of esterified PPF₁ followed by an increase of free PPF₁ was observed. Levels of esterified PPF₁ started to increase within 30 min and reached maximal levels (12 µg/g of dry weight) after 4 to 5 h (Fig. 7B). Levels of free PPF₁ increased after 3 h and reached a transient maximum (1.6 µg/g of dry weight) after 12 h (Fig. 7A).

In additional experiments, California poppy suspension cells were treated with 500 µM cupric acetate. A biphasic induction of PPF₁ levels was observed. Levels of esterified PPF₁ began to increase after a lag phase of 2 to 3 h and reached a first maximum (20 µg/g of dry weight) 6 h after copper(II) ion administration. Thereafter, PPF₁ levels declined and rose again after 14 h (Fig. 7D). Levels of free PPF₁ began to increase after a lag phase of about 4 h, reached a first maximum (2.8 µg/g of dry weight) 12 h after cupric acetate treatment. A second increase was observed after 24 h (Fig. 7D).
weight) after 9 h, declined thereafter, and increased again after 15 h (Fig. 7C).

**DISCUSSION**

**Occurrence of Prostaglandin-Like Compounds in Plants**

Since the discovery of prostaglandins in mammals, several reports have appeared describing the identification of prostanoids in plants and microorganisms (for review, see Bundy, 1985; Panossian, 1987; Hamberg and Gardner, 1992; Lamacka and Sajbidor, 1995). However, formation of prostaglandins in these organisms is difficult to rationalize since with the exception of some lower plants (algae, mosses, and ferns) and few filamentous fungi, the crucial substrate for prostaglandin synthesis, arachidonic acid, can generally not be biosynthesized by these organisms. Furthermore, prostaglandin-endoperoxide H synthase (cyclooxygenase) enzyme activity required for prostaglandin biosynthesis appears to be restricted to animals. A pathogen-inducible oxygenase with significant homology to mammalian cyclooxygenase-1 and 2 has recently been identified in tobacco (Sanz et al., 1998). However, the tobacco enzyme and its homologous enzyme from Arabidopsis have subsequently been shown to represent a novel \( \alpha \)-dioxygenase, which catalyzes conversion of \( \alpha \)-linolenic acid and other fatty acids into their 2(R)-hydroperoxy derivatives (Hamberg et al., 1999). Thus at present there exists no evidence for enzymatic formation of a prostaglandin H-like prostanoid precursor in plants. However, here we show that prostaglandin F\(_1\)-like PPF\(_1\) can be generated from linolenic acid via a nonenzymatically formed prostaglandin H-like intermediate.

**Analysis and Occurrence of PPF\(_1\)**

For GC-NICI-MS analysis of PPF\(_1\), an optimized sample preparation protocol has been established that largely removes acyclic trihydroxylated fatty acids. In addition, PPF\(_1\) could be differentiated from remaining acyclic triols by MS after catalytic hydrogenation. Nonenzymatically formed PPF\(_1\) yield a characteristic peak pattern in GC-NICI-MS chromatograms. The PPF\(_1\) signature has been found in autoxidized linolenate samples, autoxidized plant drugs, in untreated cell cultures and plants, in copper(II) ion and peroxide stressed plant cell cultures, as well as in wounded plants.

We also demonstrated that PPF\(_1\) occur in free and esterified form in plants. Esterified PPF\(_1\) are likely located in membrane lipids as has been shown for isoprostanes \( \text{F}_2 \) in mammals (Morrow et al., 1992). It has been postulated that formation of esterified isoprostanes occurs by autoxidation of esterified arachidonate within the membrane where the majority of the fatty acids resides. Free isoprostanes in turn are thought to be generated predominantly by the action of lipases on preformed isoprostane esters in membrane lipids (Waugh et al., 1997). Because the majority of linolenate in plants occurs esterified (often more than 99% [Conconi et al., 1996]), a similar scenario may be assumed for the synthesis of plant phytoprostanes (Mueller, 1998).
In the species so far analyzed, levels of esterified PPF1 were one to two orders of magnitude higher than free PPF1. Furthermore, in butyl hydroperoxide or copper(II) ion treated cells, as well as in wounded plants, levels of esterified PPF1 increased earlier and reached higher levels than free PPF1 (Fig. 7), suggesting that reactive oxygen species (ROS) initiate PPF1 synthesis in membrane lipids. However, if lipase-dependent release of PPF1s from membranes occurs in plants remains to be shown.

In an alternate manner, it may be assumed that linolenate is liberated from membrane stores in various pathophysiological situations and may serve as precursor for free PPF1. In the case of tissue injury by wounding, it has previously been shown that injury of tomato plants is accompanied by generation of ROS (Orozco-Cardenas and Ryan, 1999) and an increase of free linolenate (Conconi et al., 1996). Thus it is conceivable that ROS may catalyze not only formation of esterified PPF1, but also of free PPF1 from liberated linolenate. The data obtained so far are compatible with both concepts.

It is apparent that plants accumulate higher levels of PPF1 than PPF1. The preferential formation of PPF1 is compatible with the concept that biosynthesis of phytoprostanes occurs in a hydrophobic environment such as a membrane, which would stabilize the endoperoxide intermediate and favor PPF1, rather than PPF1, formation (Fig. 1; Table I). In addition, nonenzymatic reduction of free and membrane-bound endoperoxide intermediates to F-ring prostanooids by glutathione appears to be more rapid than rearrangement of the endoperoxide intermediates to E-ring prostanooids, at least in mammals (Morrow et al., 1998).

Isoprostanoids as Marker of oxidative stress in vivo

Perhaps the best established aspect of isoprostanoid research is the finding that isoprostanoids can be used as a quantitative index of lipid peroxidation in humans. As they are chemically stable end products of lipid peroxidation they appear to be more reliable and accurate markers of oxidative stress than other oxidation products such as malondialdehyde, especially in vivo (Roberts and Morrow, 1997). F-ring isoprostanoids are generally preferred as markers since they are more abundant and stable than the other isoprostanoids. Increased isoprostane F2 levels have been measured after intoxication and in several human disorders that are suspected to be associated with excessive generation of free radicals (Mueller, 1998; Lawson et al., 1999).

Here we show that PPF1s accumulate in oxidatively stressed plant cells. Our preliminary findings suggest that PPF1s may be useful as indicators of oxidative stress in plants. For instance, hydroperoxides and copper ions that are known to initiate lipid peroxidation (Frankel, 1998) dramatically elicit PPF1 formation in vivo (Fig. 7).

Isoprostanoids May Deteriorate Membrane Integrity Leading to Cell Death

Formation of ROS, lipid peroxidation, loss of membrane integrity, and cell death are typical features of many abiotic stresses and the hypersensitive reaction of plants against incompatible microorganisms. ROS are thought to function as a signal that may induce defense gene activation and programmed cell death. In addition, excessive formation of ROS leads to cellular damage/cell death, primarily through damage to the photosystem II reaction center and to oxidation of membrane lipids (Bowler and Fluhr, 2000).

Esterified fatty acid oxidation products in membranes may disturb membrane integrity and induce plasma leakage. In this respect, esterified F-ring isoprostanoids have been postulated to participate in membrane leak development due to their remarkably kinked geometry, as well as the repulsion by the three newly created hydroxy groups and the adjacent lipophilic fatty acids in the membrane bilayer, leading to cell death (Morrow et al., 1992; Rokach et al., 1997a; Lawson et al., 1999). Other polyhydroxylated fatty acids that may be generated in addition to phytoprostanes by autoxidation of fatty acids in membranes have, to the best of our knowledge, not yet been quantified accurately.

A major source of oxygenated fatty acids in plants appears to be lipoxygenase acting on free fatty acids. For instance, fatty acid hydroperoxides were shown to increase from 25 to 335 \( \mu g/g \) of fresh weight 30 h after application of cryptogein, an established inducer of hypersensitive reaction. It was estimated that 90% of the total fatty acid hydroperoxides were generated by lipoxygenase, whereas the remaining 10% represent membrane-bound fatty acid hydroperoxides (33.5 \( \mu g/g \) of fresh weight) formed by free radical-catalyzed oxidation of membrane lipids simultaneously with the enzymatic process (Rusterucci et al., 1999). However, it is not known at which concentration different membrane-bound oxygenated fatty acid species become lethal to cells.

Biological Activity of Isoprostanoids

Several isoprostanates have been shown to have potent biological effects on mammalian tissues. For instance, isoprostanes F2 act as broncho- and vasoconstrictors in the nanomolar concentration range, modulate platelet function, contract lymphatics, and may also induce mitogenesis (for review, see Mueller, 1998). It remains to be shown whether the structurally related inducible PPF1 exert biological activity. However, orally administered prostanooids are slowly absorbed and rapidly metabolized and, thus may possibly not be effective in mammals.

It is notable that phytoprostanes are also structurally related to enzymatically formed linolenate metabolites of the jasmonate-type. Basal levels of phytoprostanes E1 and F1 are generally more than one
order of magnitude higher than jasmonic acid levels. However, if phytoprostanes play a role in plant stress physiology has not yet been addressed. This issue remains an exciting challenge for future research.

MATERIALS AND METHODS

α-Linolenic acid (puriss. p.a.) and N,N,N,N-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were purchased from Fluka (Neu-Ulm, Germany). Oxygen-18O2 gas (99.1 atom% 18O) was obtained from Isotec (Miamisburg, OH). Silica solid phase extraction glass columns (500 mg) and silica TLC plates (Polygram Sil G/UV) were from Macherey and Nagel (Düren, Germany). Silica gel 60 (particle size 63–100 μm) for column chromatography was from Merck (Darmstadt, Germany). PFB bromide and N,N-diisopropylethylamine were from Sigma (Deisenhofen, Germany). [5,6,8,9,11,12,14,15-2H]PGF2 (specific activity of 179.00 Ci/mmol) was from NEN Life Science Products (Boston).

Preparation of PPF1 and [18O]3PPF1 by Linolenic Acid Autoxidation

Linolenic acid (5 g) was dissolved in hexane/diethyl-ether (9:1, v/v), loaded on a silica column (10 g), and eluted with hexane/diethylether (9:1, v/v) to remove any pre-formed oxygenated fatty acids. The eluate was taken to dryness in a 500-mL round bottom flask and dissolved in 50 mL of carbon tetrachloride. The flask was evaporated in vacuo and filled with 500 mL of oxygen (or oxygen-18 gas, 99.1% atom 18O in the labeling experiment). After stirring the mixture for 10 d at room temperature, the autoxidation mixture was reduced by the addition of 5 g of triphenylphosphine in 50 mL of chloroform and processed as described in “Purification of PPF1.”

Extraction of PPF1 from Plant Material

For analysis of unesterified PPF1, plant material (3 g of fresh weight or 0.3 g of dry weight) was suspended in 20 mL of cold brine containing 0.05% (w/v) of 2,6-di-tert-butyl-4-methylphenol, 20 mg of triphenylphosphine, and 0.2 mL of 1 m citric acid. [18O]3PPF1s (500 ng) were added as an internal standard. After addition of 20 mL of diethyl ether the mixture was homogenized for 3 min with a high performance disperser (Ultra-Turrax T 25, IKA-Werk, Germany) at 24,000 rpm and centrifuged (2,000g, 10 min). The ether phase was removed, taken to dryness under a stream of nitrogen, and dissolved in chloroform.

For the determination of total PPF1s (free and esterified), lipids were extracted using a modified Folch procedure. Plant material (1 g of fresh weight or 0.1 g of dry weight) was suspended in 18 mL of methanol/chloroform (2:1, v/v) containing 80 μL of 1 M citric acid, 0.115 mg of 2,6-di-tert-butyl-4-methylphenol, 20 mg of triphenylphosphine, and 500 ng of [18O]3PPF1s. After homogenization of the mixture with a high performance disperser for 1 min, 18 mL of chloroform and 8 mL of a 0.88% (w/v) potassium chloride solution were added. The lower organic phase was separated and taken to dryness. The residue was reconstituted in 2 mL of methanol containing 0.1% (w/v) 2,6-di-tert-butyl-4-methylphenol and hydrolyzed after addition of 2 mL of 15% (w/v) KOH at 40°C for 1 h. The reaction was stopped by adding 4 mL of 1 m citric acid and 32 mL of water. Lipids were extracted with diethyl ether, taken to dryness, and reconstituted in 2 mL of chloroform.

Purification of PPF1

Linolenic acid (5 g) autoxidation mixture was treated with triphenylphosphine (5 g) for 30 min. The mixture was taken to dryness, reconstituted in 50 mL of chloroform, and loaded on a silica column (4 × 21 cm, 30 g of silica gel 60). The column was washed with 600 mL of diethylether:acetic acid (99:1, v/v) and 300 mL of chloroform:acetone:acetic acid (80:20:1, v/v) to remove remaining linolenic acid, mono-oxygenated linolenate products, and triphenylphosphine. PPF1 were chromatographed isocratically with diethylether:acetone:acetic acid (70:30:1, v/v). Sixty-milliliter fractions of the eluate were taken and aliquots were analyzed by TLC (see below) for the presence of PPF1. PPF1 eluted with 60 to 480 mL of eluent.

On an analytical scale, nonesterified PPF1 from plant extracts or linolenate autoxidation mixtures were applied as a chloroform solution to a silica solid-phase extraction column (500 mg). The column was subsequently washed with 6 mL of diethyl ether:acetic acid (98:2, v/v) and 3 mL of chloroform:acetone:acetic acid (80:20:1, v/v). PPF1 were eluted with 6 mL of diethyl ether:methanol:acetic acid (90:10:1, v/v), taken to dryness, and dissolved in 2 mL of methanol.

Preparation of PPF1 Standard Solutions

A mixture of silica-purified PPF1 was further purified on a reversed phase HPLC column (LiChrospher 100 RP18e, 5-μm particle size, 8 × 250 mm; Knauer, Berlin, Germany) eluted with acetonitrile:water:acetic acid (20:80:0.1, v/v) at a flow rate of 3.5 mL/min, and detected at 204 nm (Fig. 3). Fractions of the eluate were collected and PPF1 isomers were identified by GC-EI-MS analysis after preparation of the corresponding methyl ester, TMS ether derivatives. PPF1 isomers eluting under the peaks 1 to 4 (Fig. 3) were each mixed with 500 ng of prostaglandin F1α (PGF1α) and esterified with 2,4′-dibromoacetophenone (100 μg) in 200 μL of acetonitrile containing 0.25% (w/v) diisopropylethylamine at 40°C for 90 min. The mixtures were taken to dryness, reconstituted in 2 mL of chloroform, and applied to a silica solid-phase extraction column. The column was washed with 3 mL of diethyl ether and the 4′-bromoacetophenone ester derivatives were eluted with 6 mL of diethylether:methanol (90:10, v/v). PPF1 and PGF1α derivatives were separated on a LiChrospher 100 RP18 end-capped column (5-μm particle size, 250 × 4 mm) eluted at a flow rate of 1 mL/min with a mixture of acetonitrile:water (40:60, v/v) for 15 min, followed by a gradient to 60% (v/v) acetonitrile over 15 min. The UV absorption of the 4′-bromoacetophenone moiety of the derivatives at 254
nm was used to detect the prostanoids and the amount of PPF₁ in each sample was calculated from the peak area of PPF₁ relative to that of PGF₁a.

**TLC Analysis of PPF₁**

PPF₁ and PGF₁a were chromatographed on silica TLC plates developed with chloroform:methanol:water:acetic acid (86:14:0.8:1, v/v). F-ring prostanoids (5 µg) were detected by spraying the plate with 3% (w/v) of cupric acetate in 80% (v/v) phosphoric acid followed by heating at 60°C for 5 min. Prostaglandin F-like material yielded a characteristic violet color. PGF₁a migrated as a sharp band at Rₚ 0.51, whereas PPF₁ gave several bands in the Rₚ range of 0.37 to 0.78.

**Analytical Derivatization Procedures for GC-MS Analysis**

For GC-EI-MS, silica purified PPF₁ were converted to their corresponding methyl esters by treatment with an etheral solution of diazomethane for 5 min. The solution was taken to dryness and silylated with 50 µL of BSTFA in 200 µL of chloroform at 40°C for 30 min. The mixture was taken to dryness and dissolved in hexane for GC-MS analysis.

For routine GC-NICI-MS analysis, PPF₁ obtained from silica column purification were derivatized either directly or, in the case of plant material, after catalytic hydrogenation. For hydrogenation, PPF₁ were dissolved in methanol. Adam catalyst (20 mg) was added and hydrogen gas was bubbled through the sample for 10 min. The sample was filtered, taken to dryness, reconstituted in 200 µL of chloroform, and derivatized with 10 µL of PFB bromide and 10 µL of N,N-diethyldiisopropylamine at 60°C for 30 min. The mixture was taken to dryness, reconstituted in 2 mL of chloroform, and applied to a silica solid-phase extraction column. The column was washed with 3 mL of diethyl ether. PPF₁ PFB esters were eluted with 6 mL of diethyl ether:methanol (90:10, v/v). The sample was taken to dryness and TMS ether derivatives were prepared with diazomethane for 5 min. The solution was taken to dryness and silylated with 50 µL of triethylamine at 60°C for 30 min. The mixture was taken to dryness and dissolved in hexane for GC-MS analysis.

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**GC-MS**

GC-NICI-MS was performed using a Varian 3400 gas chromatograph interfaced to a Finnigan MAT quadrupole SSQ 700 mass spectrometer. The MS source was set at 150°C and the electron energy at 70 eV. Isobutane was used as reactant gas when operating the instrument in the NICI mode. PPF₁ were analyzed on a 25 m, 0.25-µm film thickness Optima-5 column (Macherey and Nagel, Düren, Germany). The injector was set at 300°C.

For analysis of PFB ester, TMS ether derivatives of PPF₁, the column temperature was programmed from 175°C to 285°C at 30°C/min and 285°C to 300°C at 2.5°C/min.

Methyl ester, TMS ether derivatives of PPF₁ were analyzed using a column temperature program from 140°C for 1 min, 140°C to 235°C at 20°C/min, 235°C to 250°C at 1°C/min, and 250°C to 300°C at 30°C/min.

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**LITERATURE CITED**


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