Lipid hydroperoxide levels in plant tissues

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Abstract

Hydroperoxides are the primary oxygenated products of polyunsaturated fatty acids and are key intermediates in the octadecanoid signalling pathway in plants. Lipid hydroperoxides (LHPO) were determined spectrophotometrically based on their reaction with an excess of Fe²⁺ at low pH in the presence of the dye xylenol orange. Triphenylphosphine-mediated hydroxide formation was used to authenticate the signal generated by the hydroperoxides. The method readily detected lipid peroxidation in Phaseolus microsomes, senescing potato leaves and in a range of other plant tissues including Phaseolus hypocotyls (26 ± 5 nmol g⁻¹ FW), Alstroemeria floral tissues (sepals 66 ± 13 nmol g⁻¹ FW; petals 49 ± 6 nmol g⁻¹ FW), potato leaves (334 ± 75 nmol g⁻¹ FW), broccoli florets (568 ± 68 nmol g⁻¹ FW) and Chlamydomonas cells (602 ± 40 nmol g⁻¹ FW). Relative to the total fatty acid content of the tissues, the % LHPO was within the range of 0.6–1.7% for all tissue types (photosynthetic and non-photosynthetic) and represents the basal oxidation level of membrane fatty acids in plant cells. In order to relate the levels of LHPO to specific signalling pathways, transgenic potato plant lines were used in which lipoxygenase (LOX) (responsible for hydroperoxide biosynthesis) and hydroperoxide lyase (a route of hydroperoxide degradation) activities were largely reduced by an antisense-mediated approach. While the LHPO levels were similar to wild type in the individual LOX antisensed plants, basal LHPO levels, by contrast, were elevated by 38% in transgenic potato leaves antisensed in hydroperoxide lyase, indicating a role for this enzyme in the maintenance of cellular levels of LHPOs.

Key words: Lipid hydroperoxides, membrane oxidation, signalling.

Introduction

Fatty acid hydroperoxides are key intermediates in the octadecanoid signalling pathway in plants and stand at the branch point of a number of competing metabolic pathways (Gardner, 1995; Blée, 1998). Fatty acid hydroperoxides can be converted by an allene oxide synthase/cyclase route (Hamberg and Fahlstadius, 1990; Harms et al., 1995) to a family of cyclopentenone compounds collectively referred to as jasmonates, which are known to have important physiological roles in the life cycle of plants (Weber et al., 1997; Creelman and Mullet, 1997; León and Sánchez-Serrano, 1999). Hydroperoxides can also undergo chain cleavage by hydroperoxide lyase (Hatanaka, 1993; Matsui et al., 1999) generating oxoacids and volatile aldehydes, which are important for the organoleptic quality of certain plant-derived foods. The volatiles may also function as antimicrobial compounds in plant defence responses (Croft et al., 1993; Deng et al., 1993) or else activate subsets of other defence-related genes (Bate and Rothstein, 1998). In addition, hydroperoxides can serve as substrates for peroxygenases and hydroperoxide isomerase (epoxy alcohol synthase) generating products which may have roles in cutin monomer formation (Blée and Schuber, 1993). Given their central metabolic role in these various pathways it is perhaps surprising that little is known about the physiological

Abbreviations: 13S-HPODE, 13S-hydroperoxy-9Z,11E-octadecadienoic acid; BHT, butylated hydroxytoluene; FOX2, ferrous oxidation of xylenol orange version 2; TPP, triphenylphosphine; LHPOs, lipid hydroperoxides; JA, jasmonic acid.

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levels of lipid hydroperoxides (LHPO) in plants and how these are regulated. This is largely due to their instability, the often lengthy procedures required to undertaking such determinations and the requirement for expensive and dedicated equipment for detection. For these reasons, to date, the thiobarbituric acid-reactive-substances (TBARS) assay which estimates the amount of malondialdehyde (MDA), a secondary end-product of polyunsaturated fatty acid oxidation has been used as an index of general lipid peroxidation (Hodges et al., 1999), but yields little information on the levels of LHPO which are, perhaps, more relevant physiologically. In addition, MDA can only be formed from fatty acids with three or more double bonds (Halliwell and Gutteridge, 1989) and since plant tissues often contain high levels of 18:2 (Δ9,12), the TBA assay may underestimate the actual extent of peroxidation. As a necessary prerequisite to address the levels of LHPO in plants, a simple and rapid method for their estimation has been developed based upon the hydroperoxide-mediated oxidation of ferrous to ferric ions under acidic conditions (Jiang et al., 1991; Nourooz-Zadeh et al., 1994, 1996). The technique, termed the FOX2 assay, uses the ferric ion indicator dye xylene orange [o-cresolsulphonphthalein-3,3'-bis (methyliminodiacetic acid) sodium salt] which binds ferric ion to produce a coloured (blue-purple) complex with an absorbance maximum at 560 nm. The method has been applied to the study of the hydroperoxide content of plasma, low-density lipoproteins, edible oils and to monitor phosphatidylethanolamine peroxidation and shows good agreement with other independent methods of hydroperoxide determination such as chemiluminescence, and iodometric evaluation (Jiang et al., 1991; Nourooz-Zadeh et al., 1996). In all of these studies, however, no extraction of the samples was necessary prior to assay. In order to apply the method to the determination of LHPO present in plant tissues total lipids were first rapidly extracted in an acidified chloroform–methanol-based solvent system (Bligh and Dyer, 1959; Lindberg and Griffiths, 1993; Griffiths et al., 1997) and the isolated lipids assayed spectrophotometrically with FOX2 reagents. The levels of LHPO in a range of plant tissues and in transgenic potato plants with altered oxylipin metabolism are reported here to provide new insights into the levels of these compounds in plants and the implications for their roles in the octadecanoid signalling pathway.

Materials and methods

Ammonium ferrous sulphate, butylated hydroxytoluene (BHT), xylene orange [o-cresol-sulphonphthalein-3,3'-bis (methyliminodiacetic acid sodium salt)], catalase, triphenylphosphate (TPP) and 18:2 were purchased from Sigma-Aldrich (Poole, Dorset, UK). All reagents were of the highest purity available.

Preparation of FOX2 reagent

FOX2 reagent was prepared according to a method described previously (Nourooz-Zadeh et al., 1995) by dissolving xylene orange (Sigma, UK) and ammonium ferrous sulphate in 250 mM HSO₄ to final concentrations of 1 mM and 2.5 mM, respectively. One volume of this concentrated reagent was added to 9 vols of HPLC grade methanol containing 4.4 mM BHT to make the working reagent which comprised 250 μM ammonium ferrous sulphate, 100 μM xylene orange, 25 mM HSO₄, and 4 mM BHT in 90% (v/v) methanol.

Preparation of fatty acid hydroperoxides

13S-hydroperoxy-9Z, 11E-octadecadienoic acid (HPODE) was enzymatically synthesized using soybean lipoygenase (Sigma, lipoygenase type 1-S) essentially according to the method of Gardner (Gardner, 1997). Product formation was monitored spectrophotometrically at 234 nm and the purity of the eluting fractions was determined at the same wavelength by HPLC (Hewlett-Packard 1100 series chromatograph) on a 5 μm Partisil column (Fisons chromatography, Leicester, UK) using isocratic elution in 0.75% ethanol in n-hexane at a flow rate of 4 ml min⁻¹ (Chan and Levett, 1977).

Plant material

Potato plants (Solanum tuberosum cv. Desiree) were grown in soil in the greenhouse at 23 °C under a 16/8 light/dark regime. Transformed plants are described elsewhere (Royo et al., 1999). Selected lines were vegetatively propagated in the greenhouse either by tuber sowing or by explant cutting. Peruvian Lily (Alstroemeria peruviana cv. Samora) floral tissues (sepal and petals) were obtained as freshly harvested cymes from a local commercial grower. Broccoli heads (Brassica oleracea var. italica cv. Marathon) were harvested from field-grown plots at Horticulture Research International (Wellesbourne, UK) during the summer of 1999. Chlamydomonas reinhardtii cell wall-less strain (cw-15) was obtained from Chlamydomonas Genetics Center, Duke University, Durham, USA. The cells were grown in liquid TRIS/acetate/phosphate medium containing Hutners trace elements (Harris, 1988), under fluorescent lighting in a Gallenkamp (USA) orbital incubator at 28 °C and shaking at 200 rev min⁻¹. Dwarf French bean (Phaseolus vulgaris cv. Tendergreen) were purchased from Kings Wholesale Seed Merchants and Growers (Kelvedon, UK). Seeds were imbibed in water overnight and then sown on absorbent paper in plastic trays and allowed to germinate in the dark for 6 d at which stage the hypocotyls were harvested.

Preparation of Phaseolus microsomes

6-d dark-grown Phaseolus hypocotyls were harvested and homogenized with a pestle and mortar in 3 vols (w/v) of TRIS-HCl (100 mM, pH 8.5). The homogenate was filtered through a double layer of Miracloth (Calbiochem, USA) and centrifuged at 20000 g for 20 min. The resulting supernatant was removed and centrifuged at 105000 g for 50 min. The microsomal pellet obtained was resuspended in TRIS-HCl (50 mM, pH 8.5) and stored at −80 °C until required.

Analytical procedures

Total lipids were rapidly extracted from tissues by a modification of the previously used method (Bligh and Dyer, 1959) according to Griffiths et al. (Griffiths et al., 1997). All procedures were performed in dim light at 4 °C using chilled solvents (containing BHT, 0.01% w/v) and glassware. Tissues (approximately 0.2 g fresh weight) were homogenized with a pestle and mortar.
containing 0.15 M acetic acid (1 ml) and chloroform/methanol (1:2 v/v; 7.5 ml) for approximately 2 min and transferred to culture tubes (Pyrex, UK). The pestle and mortar were rinsed with chloroform (2.25 ml) and combined with the extract to which was added distilled water (2.25 ml). Phase separation was facilitated by low speed centrifugation and the lower chloroform (CHCl$_3$) phase containing the lipids was removed and aliquots dispensed into amber vials (Hewlett Packard, USA) and evaporated under N$_2$. Vials were capped and stored on ice until all samples had been evaporated to dryness. Samples were resuspended in HPLC grade methanol either in 100 µl for samples without TPP or in 90 µl methanol to which were added 10 µl TPP (25 mM in methanol). Samples were allowed to incubate at room temperature for 30 min in the dark and then for a further 30 min following the addition of the working FOX2 reagent. Absorbances were determined spectrophotometrically at 560 nm and the concentration of LHPOs determined using a molar absorption coefficient derived for standard linoleate hydroperoxide (ε = 6.0 x 10$^4$ M$^{-1}$ cm$^{-1}$, Gay et al., 1999).

**Calibration of FOX2 reagent with 13S-HPODE**

The detection limits of the FOX2 assay were determined using a concentration range of 13S-HPODE from 0.75 µM up to 150 µM in methanol, TPP (in methanol, final concentration 2.5 mM) was added to one series of samples to reduce the LHPOs to their corresponding non-reactive hydroxide derivatives and was used to authenticate the signal generated in the samples minus TPP following the addition of FOX2 reagents. Methanol plus TPP controls had negligible absorbance. The results (not shown) indicate that the FOX2 assay can detect up to 75 µM 13S-HPODE ($R^2$ = 0.983). The curvilinear data fits a single exponential equation where the concentration = \(-49.427 \times \log_{(2.9011-\text{abs})}/2.9328.

**Effect of butylated hydroxytoluene (BHT) addition**

BHT is routinely added to organic solvents as an antioxidant to limit lipid peroxidation. To determine whether this compound affected the level of LHPOs detected in the FOX2 assay an attempt was made to reduce the possible artefactual generation of LHPOs in extracts by adding BHT at a concentration recommended for lipid extraction of 10 mg per 100 ml of solvent (Christie, 1982). The potential problems associated with photo-oxidation and reactive-specie damage occurring in chlorophyllous tissue was studied using potato leaf lipid extracts prepared with or without BHT added to the solvents. In samples without BHT added to the solvents, the LHPO content was 745 ± 117 nmol g$^{-1}$ FW and with BHT was 410 ± 47 nmol g$^{-1}$ FW (n = 4). The results show that addition of BHT to the solvents reduced the apparent LHPOs content by 45% and so was routinely added to solvents in all subsequent determinations. Addition of higher levels of BHT had no further effect (data not given).

**Pigment interference**

The treatment of lipid extracts with TPP prior to incubation with FOX2 reagents was also considered as a possible source of error in the overall assay procedure due to non-specific degradation of pigments resulting in an apparent change in $\Delta_{\text{abs}}$. To investigate this we performed spectral scans (between 220–700 nm) of *Alstroemeria* and potato leaf lipids dissolved in methanol prior to and following the addition of TPP. In both cases the lowest absorbance was between 520–580 nm. Pigment absorbance was high between 430–480 nm (corresponding to carotenoids) and between 647 and 664 nm characteristic of chlorophylls $b$ and $a$. Addition of TPP had no effect on the $\Delta_{\text{abs}}$ values (data not shown). The signal generated following addition of FOX2 reagents is, therefore, dependent on these reagents and is not generated as a non-specific alteration in the $\Delta_{\text{abs}}$ caused by TPP.

**Recovery and stability of 13S-HPODE following solvent extraction**

Lipid extracts were quantified as their fatty acid methyl ester derivatives obtained by transesterification performed in 2.5% (by vol.) sulphuric acid in anhydrous methanol (2 ml) and separated on a gas liquid chromatograph equipped with a flame ionization detector (FID). Hexadecanoic acid (17:0) was used as the internal standard and separation was achieved on a 10% DEGS CW AW column (Jones Chromatography, UK) at 170 °C with nitrogen (30 ml min$^{-1}$) as the carrier gas.

**Protein and chlorophyll determinations**

Protein determinations were made using the standard assay protocol with bicinchinonic acid (BCA, Pierce and Warner, UK) with bovine serum albumin as a standard. Chlorophyll was determined in the chloroform (lipid) extracts using the equations of Wellburn (Wellburn, 1994).

**Results**

**Application of the FOX2 method for the determination of LHPOs in microsomal membranes**

Microsomal membranes have been used in many studies on lipid peroxidation (Poli et al., 1985; Jasinska et al., 1996; Buko et al., 1999). In order to determine whether the FOX2 assay could detect changes in LHPO, Phaseolus microsomes were prepared and the LHPO content determined in samples incubated in buffer at 4 °C or 18 °C (Table 1). LHPOs could not be detected in freshly thawed microsomes or in samples maintained at either temperature for 1 h. After 2 h, however, hydroperoxides were detected at a level 8-times higher at 4 °C than at 18 °C. After 4 h, the content of hydroperoxides doubled at 4 °C.
and were still 5-times higher than those at 18 °C. Since peroxidation is higher at elevated temperatures the accumulation of higher levels of LHPOs at 4 °C is most likely related to their greater thermal stability at this temperature. The results indicate that the FOX2 assay can detect changes in LHPO content in such experimental systems.

**Fatty acid profiles, LHPO and chlorophyll content of various tissues**

It was anticipated that photosynthetic tissues may contain higher levels of LHPO than non-photosynthetic tissues resulting from reactive oxygen species-damage during the extraction process. In order to determine whether this was so, a range of plant tissues was chosen to investigate this possibility, namely non-photosynthetic dark-grown hypocotyls, floral tissues, leaf tissue, and the photosynthetic unicellular alga, *Chlamydomonas*. The fatty acid profile of total lipids extracted from the various tissues is given in Table 2. Palmitate (16:0), stearate (18:0) oleate (18:1 Δcis,9), linoleate (18:2 Δcis,cis,9,12) and linolenic acid (18:3Δcis,9,12,15) are ubiquitously present. The series of 16 carbon unsaturated fatty acids up to the level of hexadecatetraenoic acid (16:3 Δall cis,7,10,13) was also present in all tissues except *Phaseolus* hypocotyls. In *Chlamydomonas*, γ-linolenic acid (γ-18:3 Δall cis,6,9,12) and stearidonic acid (18:4 Δall cis,6,9,12,15) were also present. In all tissues the predominant polyunsaturated fatty acids are 18:2 and γ-linolenic acid (18:3 Δall cis,9,12,15). The results expressed as fatty acid content g⁻¹ FW shows that tissues vary significantly in their lipid content (Table 3). The highest lipid content is in *Chlamydomonas* cells which is over 2, 3, and 28-fold higher than broccoli, potato leaf, and dark-grown *Phaseolus* hypocotyls, respectively. The fatty acid content in *Alstroemeria* floral tissues is about one-third of that in potato leaf. The content of LHPOs expressed g⁻¹ FW also varies widely from tissue to tissue (from 26–602 nmol g⁻¹ FW). The highest levels are observed in those tissues, which also have the highest lipid content. (e.g. *Chlamydomonas*) while those with the lowest lipid content (*Phaseolus* hypocotyls) have the lowest LHPOs. The relationship between the fatty acid content and the LHPO content is not stoichiometric, but generally the higher the lipid content of the tissue the higher the LHPO content and, by expressing the data as % total fatty acids oxidized, it is evident that the levels are in the range of 0.6–1.7% for all tissue types examined. In addition, the level of LHPOs are not related to the chlorophyll content of the tissues (compare the % total oxidized fatty acids in *Phaseolus* (1.0%) which contains no chlorophyll with *Chlamydomonas* (0.8% total oxidized fatty acids) which contains 7.9 mg chlorophyll g⁻¹ wet weight cells). Thus, the relative LHPO levels are similar in photosynthetic and non-photosynthetic tissues indicating little reactive oxygen species-damage during the lipid extraction process of chlorophyllous tissues.

**Determination of LHPO content in the leaves of transgenic potatoes with altered LHPO metabolism**

A range of potato plants with altered expression of genes encoding proteins which catalyse specific steps at the start of the octadecanoid biosynthetic pathway have been generated (Royo et al., 1999; J Leon, G Vancanneyt and JJ Sanchez-Serrano, unpublished results). These include plants antisensed with respect to (1) lipoxygenase (anti-LOX H1 and anti-LOX H3) which catalyses the dioxygenation of unsaturated fatty acids at their C-13 position and (2) fatty acid hydroperoxide lyase (anti-HPL), the

<table>
<thead>
<tr>
<th>Table 1. Lipid hydroperoxide content of Phaseolus microsomal membranes incubated at 18 °C and 4 °C</th>
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<tr>
<td>A 1 ml suspension of microsomal membranes in TRIS-HCl buffer (50 mM pH 8.0) containing 9 mg ml⁻¹ protein were incubated at 18 °C and 4 °C. At the times indicated aliquots (equivalent to 90 μg protein) were removed and the LHPO content determined in lipid extracts using the FOX2 assay. Values presented are means ± SD (n = 3). nd = Not detected.</td>
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<tr>
<td>Time (h)</td>
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<td>0</td>
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<thead>
<tr>
<th>Table 2. Fatty acid composition of the tissues used for LHPO determination</th>
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<tr>
<td>Lipid extracts were transmethylated and analysed as their fatty acid methyl ester derivatives by GC and quantified using heptadecanoic acid as an internal standard. Values are mean ± SD (n = 4); *mean ± SD (n = 14).</td>
</tr>
<tr>
<td>Tissue type</td>
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<td>-------------</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Phaseolus</td>
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<tr>
<td>Hypocotyls</td>
</tr>
<tr>
<td>Sepals</td>
</tr>
<tr>
<td>Petals</td>
</tr>
<tr>
<td>Potato leaf</td>
</tr>
<tr>
<td>Broccoli florets</td>
</tr>
<tr>
<td><em>Chlamydomonas</em></td>
</tr>
</tbody>
</table>
Lipid hydroperoxides in plants

**Table 3. Lipid hydroperoxide, fatty acid and chlorophyll content of various plant tissues**

Lipids were extracted and quantified by GC and the hydroperoxide (LHPO) content determined by the FOX2 assay. Values presented are the means ± SD (n=6); * values are mean ± SD (n=14).

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Chlorophyll µg−1 FW</th>
<th>nmol fatty acid g−1 FW</th>
<th>nmol LHPO g−1 FW</th>
<th>% total fatty acids oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phaseolus (hypocotyls)</td>
<td>nd</td>
<td>2 ± 0       62 ± 247</td>
<td>26 ± 5</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Alstroemeria</td>
<td></td>
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<tr>
<td>Sepals</td>
<td>142 ± 2</td>
<td>6 ± 291 ± 332</td>
<td>66 ± 13</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Petals</td>
<td>193 ± 17</td>
<td>8 ± 062 ± 160</td>
<td>49 ± 6</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Potato (leaf*)</td>
<td>1 ± 307 ± 256</td>
<td>22 ± 458 ± 3216</td>
<td>334 ± 75</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Broccoli (florets)</td>
<td>608 ± 145</td>
<td>33 ± 921 ± 5988</td>
<td>568 ± 68</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Chlamydomonas</td>
<td>7 ± 941 ± 291</td>
<td>73 ± 744 ± 4068</td>
<td>602 ± 40</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>

**Table 4. Lipid hydroperoxide content in leaves of transgenic potato plants antisensed to lipoygenase or hydroperoxide lyase**

Lipids were extracted from potato leaves and the LHPO content determined using the FOX2 assay. Results are the mean ± SD (n=6).

<table>
<thead>
<tr>
<th>Transgenic potato line</th>
<th>nmol LHPO g−1 FW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type*</td>
<td>407 ± 21</td>
</tr>
<tr>
<td>Anti-HPL</td>
<td>566 ± 16</td>
</tr>
<tr>
<td>Anti-LOXH1</td>
<td>426 ± 25</td>
</tr>
<tr>
<td>Anti-LOXH3</td>
<td>399 ± 15</td>
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*Wild type values are based on plants of equivalent stature and age to those of the antisensed lines and not those determined in Table 3 which is based on a larger and more diverse population of plants.

**Discussion**

Fatty acid hydroperoxides play central roles in cell signaling and the generation of defence compounds in plants. However, to date, little information is available on the level of these compounds in plant tissues and no simple method for their detection has been reported. Of primary concern during the course of this study was the desire not to generate LHPOs artefactually in tissue extracts and to ensure the stability of the putative hydroperoxides once isolated. Lipid hydroperoxides are exceptionally stable under favourable conditions, such as low temperature, dilute solution, the presence of antioxidants and the absence of catalysts such as iron salts (Gardner, 1987). The determination of LHPOs in photosynthetic tissues was viewed as potentially more problematical as light absorption by species other than lipids can initiate the formation of free radicals in sensitized photosynthesis (Chan and Cotton, 1987). Originally it had been anticipated that photosynthetic tissues may contain higher levels of LHPO than non-photosynthetic tissues resulting from reactive oxygen species-damage during the extraction process. In practice, it was found that, indeed, photosynthetic tissues do contain higher levels of LHPO than non-photosynthetic tissues. However, it is important to relate the hydroperoxide content to the total lipid content of the tissue. When the data are expressed in this way it is evident that the relative LHPO content is within the range of 0.6–1.7% for all tissue types (photosynthetic and non-photosynthetic) and this value represents the basal level of oxidation of membrane polyunsaturated fatty acids in plant tissues detected by this method. The mechanisms by which LHPO levels are maintained below 2% of total fatty acids is, at present, unclear. During senescence in *Alstroemeria* sepals, for example, this basal value rises from 1% up to 4% suggesting an impairment of mechanisms which limit lipid oxidation (M Leverentz and G Griffiths, unpublished observations). In senescing potato leaves the level of LHPO increases when the chlorophyll content is <25% of mature leaves. Interesting data were also obtained from the determination of LHPO content of leaf tissue from transgenic potato plants blocked at specific steps in the oxylipin pathway. In plants
in which the hydroperoxide lyase activity was reduced by 95% (G Vancanneyt et al., unpublished results) the tissues showed an accumulation of LHPOs of nearly 40% more than the wild type (representing an increase from 1.5% to 2.1% LHPO of total fatty acids) without displaying an altered phenotype. Since LHPOs are deleterious to membrane function (Lesham, 1992) mechanisms for their efficient removal would be envisaged. Recent evidence from studies on salt-stressed citrus suggests that phospholipid hydroperoxide glutathione peroxidase plays a role in LHPO detoxification (Avsian-Kretchmer et al., 1999) and this enzyme also appears to be an important route in animal tissues (Yagi et al., 1996). How LHPO levels are regulated in the anti-HPL plants is at present unclear. In this regard, however, analyses of the volatiles emitted by the leaves of these transgenics indicate that, in this case, LHPOs may degrade by an autoxidation mechanism (G Vancanneyt et al., unpublished observations). Antisense-mediated depletion of a specific 13-lipoxygenase (LOX H3) has been shown to reduce wound-induction of pin genes, increase tuber yields and favour larger weight gains of insect pests feeding on the transgenic plants (Royo et al., 1999). However, wound-induced JA levels were similar in wild type and LOX H3-depleted plants, suggesting that the involvement of this particular 13-lipoxygenase isoform in plant defence is not related to the bulk production of wound-induced JA. In this study it was found that LOX H3 antisense plants have levels of LHPO similar to wild type. A second 13-LOX gene product has been identified in potato leaves (LOX H1, Royo et al., 1996). Co-suppression of LOX H1 gene expression results in plants with marked morphological alterations. These plants have severely reduced levels of C6-aldehyde products of the hydroperoxide lyase pathway (G Vancanneyt et al., unpublished observations) and again show LHPO levels nearly identical to wild type, whereas one would have predicted a decrease in the endogenous LHPO levels in these plants. However, in recent metabolic studies using potato leaf homogenates, Hamberg (Hamberg, 1999) has shown that the major lipoxygenase activity using linoleic acid as substrate is a 9-LOX-catalysed oxygenation producing 9(S)-hydroperoxide in a ratio of 95:5 over the 13-LOX reaction. Since the FOX2 assay does not distinguish between positional isomers of oxygenated fatty acids, the level of the 9-LOX product is likely unaffected in both the 13-LOX antisense lines (H1 and H3) and, therefore probably accounts for the maintenance of the LHPO in these lines similar to the wild type determined in this study.

It is interesting to note that following mechanical damage the JA concentration increases in potato leaves from basal levels of about 2 nmol g⁻¹ FW to 10–12 nmol g⁻¹ FW over 6 h and by 24 h starts to decline (Martin et al., 1999). The level of LHPOs determined in the present study indicates a basal level of 334 nmol g⁻¹ FW. Since 18:3 is the major fatty acid in potato leaves, it is likely that the major hydroperoxides will be also of this fatty acid. Thus a substantial pool of potential 18:3 hydroperoxide is present, in theory, to support JA synthesis following wounding. Whether unesterified fatty acid hydroperoxides or fatty acid hydroperoxide esterified in complex lipids serve as substrates for the initiation of the jasmonate signalling pathway is not known and no lipase with such a role has been identified to date. However, it is evident from the present study that the LHPO pool (334 nmol g⁻¹ FW) is larger than the total unesterified fatty acid (UFA) pool (approximately 1% of total fatty acids, equivalent to 225 nmol g⁻¹ FW, G Griffiths, unpublished observations) indicating that the bulk of the hydroperoxides are esterified in acyl lipids. Currently, the cellular location, origin or isomeric composition of this LHPO pool is not known or whether it is available for participation in signal transduction. Since LOX H3 plants are compromised in the wound response the current data would indicate that the pool of LHPO detected in the present study cannot be readily utilized for this purpose. Recently, the wound response in potato plants depleted of fatty acid ω3-desaturase has been characterized by an antisense approach. These plants showed reduced levels of α-18:3 and a lower wound-induced pin 2 expression (Martin et al., 1999). Again, however, even in the most severely antisensed ω3-desaturase lines there was still a 180-fold excess of potential α-18:3 substrate present in the leaf lipids to support JA synthesis. This observation taken together with the excess of LHPOs reported here indicates that the products to be used in signalling are effectively metabolically channelled in mediating the wound response in plants and that bulk pools of LHPO (and α-18:3) are not readily available for this purpose. The method of LHPO determination reported here is a rapid and simple technique and may be useful for the determination of LHPO in tissues where the levels of these compounds may be expected to vary in plants grown under conditions of abiotic and biotic stress or during the course of senescence in plant organs.

Acknowledgements

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