Fruit-specific lipoxygenase suppression in antisense-transgenic tomatoes

Allen Griffiths a, Samantha Prestage b, Rob Linforth b, Jianliang Zhang a, Andy Taylor b, Don Grierson a,*

a Plant Science Division, School of Biological Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK
b Food Science Division, School of Biological Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK

Received 12 April 1999; accepted 30 July 1999

Abstract

To determine the importance of the enzyme lipoxygenase (LOX) in the generation of volatile C6 aldehyde and alcohol flavour compounds, two antisense LOX genes were constructed and transferred to tomato plants. The first of these constructs (p2ALX) incorporated the fruit-specific 2A11 promoter [Van Haaren, M.J.J., Houck, C.M., 1993. A functional map of the fruit-specific promoter of the tomato 2A11 gene. Plant Mol. Biol. 21, 625–640] and a 1.2 Kb antisense fragment of the cDNA of PTL1 (TomloxA) [Ferrie, B.J., Beaudoin, N., Burkhart, W., Bowsher, C.G., Rothstein, S.J., 1994. The cloning of two tomato lipoxygenase genes and their differential expression during tomato fruit ripening. Plant Physiol. 106, 109–118]; no terminator was included. The second construct (pPGLX) consisted of the ripening-specific polygalacturonase (PG) promoter and terminator [Nicholass, F.J., Smith, C.J.S., Schuch, W., Bird, C.R., Grierson, D., 1995. High levels of ripening-specific reporter gene expression directed by tomato fruit polygalacturonase gene-flanking regions. Plant Mol. Biol. 28, 423–435] and a 400 bp antisense fragment of the tomato cDNA of PTL1 (TomloxA). Both constructs included the highly conserved LOX region (approximately 110 bp) shared by all plant and mammalian lipoxygenase genes. Reduced levels of endogenous TomloxA and TomloxB mRNA (2–20% of wild-type) were detected in transgenic fruit containing the p2ALX construct compared to non-transformed plants, whereas the levels of mRNA for a distinct isoform, TomloxC, were either unaffected or even increased. The pPGLX construct was much less effective in reducing endogenous LOX mRNA levels. In the case of the p2ALX plants, LOX enzyme activity was also greatly reduced compared with wild-type plants. Analysis of flavour volatiles, however, indicated that there were no significant changes. These findings suggest that either very low levels of LOX are sufficient for the generation of C6 aldehydes and alcohols, or that a specific isoform such as TomloxC, in the absence of TomloxA and TomloxB, is responsible for the production of these compounds. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Lipoxygenase; Flavour; Fruit ripening; Tomato; Gene expression

* Corresponding author. Tel.: +44-115-951-6333; fax: +44-115-951-6334.
E-mail address: donald.grierson@nottingham.ac.uk (D. Grierson)

0925-5214/99/$ - see front matter © 1999 Elsevier Science B.V. All rights reserved.
PI: S0925-5214(99)00051-4
1. Introduction

Both plant and mammalian lipoxygenases (LOX) catalyze the hydroperoxidation of polyunsaturated fatty acids containing a cis,cis-pentadiene structure. The reaction intermediates in plants are unsaturated fatty acid hydroperoxides (HPOs) which, via the sequential action of hydroperoxide lyases (HPO-lyases), give rise to the aroma volatile compounds responsible for the fresh tomato flavour, notably the C6 aldehydes hexanal and hexenal (Buttery et al., 1989; Riley et al., 1996). These characteristic volatile flavour compounds are typically released following breakage or disruption of the tissue. There appear be two groups of HPO-lyases, cleaving either 9-(S)-HPOs or 13-(S)-HPOs and generating either two C9 fragments or a C6 and a C12 fragment respectively (Hatanaka, 1993). In tomato fruit it appears that the majority of the HPOs formed are the 9-isomers, although it is the much smaller proportion of the 13-isomers formed which appears to be metabolised further (Galliard and Matthew, 1977; Regdel et al., 1994; Smith et al., 1997) (see Fig. 1). Linoleic and linolenic acid are the main LOX substrates in tomato fruit, with the action of the 13-lyase on the 13-HPO products of these substrates giving rise to hexanal and hexenal, respectively (Galliard and Matthew, 1977; Galliard et al., 1977).

Both the biochemical and immunological characterisation of soybean, rice and pea LOX have been well documented (Gardner, 1991; Siedow, 1991), but the physiological and biochemical significance of LOX in plants remains equivocal. The proposed roles for LOX in various aspects of plant growth and development have included the biosynthesis of regulatory molecules (Bell et al., 1995), senescence (Todd et al., 1990) and wound and stress responses (Heitz et al., 1997). Using a transgenic approach, a physiological role for the chloroplast-located LOX isozyme, LOX2, in wound-induced jasmonic acid (JA) production has been proposed in Arabidopsis thaliana (Bell et al., 1995).

Four tomato LOX have previously been reported (Ferrie et al., 1994; Kausch and Handa, 1995, 1997; Heitz et al., 1997). Three of these tomato cDNAs have been shown to be expressed during fruit ripening, TomloxA and TomloxB (Ferrie et al., 1994), U13681 (Kausch and Handa, 1995, 1997), and TomloxC (Heitz et al., 1997). The clone U13681 (Kausch and Handa, 1995, 1997) and TomloxB (Ferrie et al., 1994) are identical. In the case of the fourth clone TomloxD, expression in fruit is very low (Heitz et al., 1997). Two of these tomato LOX TomloxA and TomloxB show 74% homology at the DNA level and 72% identity at the amino acid level (Ferrie et al., 1994), whereas the similarity of TomloxA and TomloxB to TomloxC is approximately 40%. An antisense approach to down-regulate the expression of the endogenous LOX mRNA in tomato fruit was adopted in the present study to investigate the role of LOX in fruit development. Taking into account the fact that LOX share several main regions of amino acid sequence homology (Siedow, 1991; Ferrie et al., 1994), it was anticipated that it might be possible to down-regulate TomloxA and TomloxB with one construct. A 38 amino acid region, which is highly conserved, contains 13 completely conserved residues and these are common to both TomloxA and TomloxB (Steczko et al., 1992; Ferrie et al., 1994). In this particular region there are five conserved histidine residues and it is believed that these may serve as iron ligands to the enzyme active site (Siedow, 1991; Steczko et al., 1992). In the current study,
the antisense constructs were designed to target this particular region of the LOX mRNA. We report on the generation of transgenic antisense LOX tomato fruit and their analysis in terms of mRNA expression studies, LOX enzyme activity and aroma volatile analysis.

2. Materials and methods

2.1. Screening of a cDNA library

Screening of an early ripening tomato cDNA library (Picton et al., 1993), using a potato LOX cDNA \textit{lox1;St1} (Casey, 1995), resulted in the identification of three previously published tomato LOX clones. PTL1 was homologous to \textit{TomloxA} (Ferrie et al., 1994); PTL2 was identical to \textit{TomloxB} (Ferrie et al., 1994) and a clone designated U13681 (Kausch and Handa, 1995), but PTL2 lacked 300 nucleotides from the 5' end; LOX3 which was not full length at 1.2 Kb was homologous to the 3' region of \textit{TomloxC} (Heitz et al., 1997). PTL1, PTL2 and LOX3 will be referred to henceforth as \textit{TomloxA}, \textit{TomloxB} and \textit{TomloxC} respectively.

2.2. Construction of antisense LOX transformation constructs using the 2A11 fruit specific promoter and the polygalacturonase promoter

All molecular cloning procedures were carried out using standard methods (Sambrook et al., 1989). The 2A11 promoter described by Van Haaren and Houck (1991, 1993), was obtained via polymerase chain reaction (PCR) (supplied by Dr Sumant Chengappa, Unilever Research, Colworth House). The construct pA2LX was assembled to generate an antisense transgene to down-regulate the endogenous \textit{TomloxA} and \textit{TomloxB} mRNA (Fig. 2A). A partial length 3' sequence of the cDNA of \textit{TomloxA}, in the antisense orientation, was assembled by ligating a 1.2 Kb KpnI/ClaI fragment of \textit{TomloxA} in front of the 2A11 promoter at the KpnI/ClaI site contained in the pBluescript derived vector pBS2A11. The presence and orientation of the \textit{TomloxA} insert was confirmed by restriction enzyme analysis and PCR, using primers specific to the highly conserved region of the LOX sequence and the T7 promoter of pBluescript. These approaches confirmed that the LOX sequence was in the antisense orientation with respect to the 2A11 promoter. No terminator was incorporated into this construct. This expression cassette containing the 2A11 promoter and LOX insert was then released by a KpnI/BamHI digest to give a 5.2 Kb fragment. This fragment was then ligated into the binary transformation vector BIN19 (Bevan, 1984) at the KpnI/BamHI site to give p2ALX. Colonies were checked for the presence of the expression cassette by blotting onto Hybond-N + membranes and probed using the original \textit{TomloxA} KpnI/ClaI insert as a probe. Further confir-
mation of the presence and orientation of the 2A11 promoter and the LOX antisense insert was achieved by restriction enzyme analysis and plasmid Southern analysis.

The second construct, pPGLX, designed to down regulate endogenous LOX mRNA expression utilised the 4.8 Kb polygalacturonase (PG) promoter (Nicholass et al., 1995), along with a partial 3’ antisense orientated sequence of Tom-loxA and the 1.8 Kb PG terminator (Fig. 2B). The PG promoter and terminator had previously been assembled into the binary vector pBIN19 (Supplied by Dr Colin Bird and Dr Rachael Drake, Zeneca Plant Science) and was designated pRD12. A 396 bp XhoI/SpeI fragment of Tom-loxA was assembled in the antisense orientation in front of the PG promoter at the XhoI/SpeI site of the pRD12 vector. Clones containing the insert were identified by restriction enzyme analysis, followed by plasmid Southern analysis and also by PCR using primers specific to the highly conserved LOX region and the PG promoter.

2.3. Plant transformation

For plant transformations the binary vectors were introduced into competent cells of Agrobacterium tumefaciens strain LBA4404 (Bevan, 1984). The transformation of tomato cotyledons (Lycopersicon esculentum cv Ailsa Craig) involved a standard transformation protocol (Bird et al., 1988). Successfully transformed plant material with roots was regenerated from callus grown on 100 µg/ml kanamycin and then transferred to the glasshouse. These plants were later potted up into compost (Levington M2) and grown under identical glasshouse conditions used for non-transformed control plants.

2.4. RNA isolation and Northern analysis

Tomato fruit pericarp tissue was harvested from the combined pericarp of two to three fruit, frozen in liquid nitrogen and then stored at −80°C until required. Total RNA was extracted from approximately 10 g of the pericarp tissue. The extraction, quantification, blotting, hybridisation and probe synthesis protocols were as described by Griffiths et al. (1999). Hybridisation was at 42°C in 50% (v/v) deionised formamide, 1% (w/v) SDS, 1 M NaCl, 10% (w/v) dextran sulphate and 100 µg/ml salmon sperm DNA.

In addition to using autoradiography to analyse the signal intensity, the membranes were quantified directly using a Packard InstantImager™ 2024 radioanalytical imaging detector and the data analysed using InstantImager™ software.

2.5. DNA isolation and Southern analysis

Genomic DNA was extracted according to the protocol of Bernatzky and Tanksley (1986), except that frozen leaf samples (2–3 g) were initially ground in liquid nitrogen using a pestle and mortar, followed by the addition of extraction buffer. The isolated DNA was digested with the appropriate restriction enzymes and fractionated on a 1.0% agarose gel and blotted onto Gene Screen Plus membranes (Gene Screen, Du Pont). Prehybridisation and hybridisation conditions were carried out at 65°C as described in the manufacturer’s instructions (Gene Screen, Du Pont).

2.6. Extraction and assaying of LOX activity

Fruit samples were harvested at various stages of development from breaker to 7 days post-breaker. Pericarp tissue was diced and frozen immediately in liquid nitrogen before storage at −80°C until required. For the LOX assay 12 g of pericarp tissue was thawed and extracted using a pestle and mortar in 12 ml of 0.1 M phosphate buffer (pH 6.5), including 0.1% (w/v) Triton X-100 and 1 mM EDTA. LOX enzyme activity measurements were made using a Clark-type oxygen electrode, where oxygen consumption was measured in the presence of the substrate linoleic acid as previously described (Smith et al., 1997).

2.7. Volatile sampling

2.7.1. Headspace sampling

Individual tomatoes (28–66 g) were placed in a plastic stomacher bag and an internal standard (0.025 mg of 2-octanone in 100 µl water) was
added. Tomatoes were macerated for 1 min by the action of paddles in the stomacher machine (Seward M50-110, London, UK). After maceration, headspace gas (160 ml) was collected onto a Tenax trap (Unijector, SGE, Milton Keynes, UK) for 1 min using a vacuum pump (Charles Austin, B100 SEC). The trap was then removed and the volatiles analysed by GC-MS as described below.

2.7.2. Chromatographic conditions

Tenax traps were desorbed in a headspace injector (Unijector; SGE) connected to a Hewlett Packard 5890 Series II gas chromatograph (column head pressure 18 psi, helium carrier gas). The volatiles were desorbed from the traps (3 min, 240°C) and cryofocused onto a 400 mm region of the column (BP-1, 25 m × 0.22 mm ID, 1 μm film thickness; SGE). After desorption, the column was held at 40°C (2 min), then temperature programmed from 40 to 106°C at 4°C/min and from 106 to 145°C at 15°C/min. Compounds were detected using an MD 800 mass spectrometer operating in the m/z range 30–150 (Fisons Scientific, Manchester, UK).

2.7.3. Data analysis

The relative amounts of compounds were determined by measuring the peak areas of characteristic ions for the volatiles of interest (hexanal m/z 92; (Z)-3-hexenal m/z 98; (E)-2-hexenal m/z 83; 2-octanone m/z 58). Peak areas for each sample were corrected by reference to the internal standard and expressed as peak area of volatile per 50 g fresh weight.

3. Results

3.1. LOX mRNA expression in transgenic plants

mRNA expression analysis of a fruit ripening series, using TomloxA, TomloxB and TomloxC as probes is shown in Fig. 3. In the case of TomloxA maximum expression occurs between the mature green and breaker stages of fruit development. In contrast, TomloxB and TomloxC exhibit maximum expression at or around the breaker to 3 days post-breaker stage of fruit development (Fig. 3). Nineteen pPGLX antisense plants were analysed at identical stages of fruit development. No major reductions in endogenous LOX mRNA levels were apparent in fruit from plants transformed with the pPGLX construct compared with fruit samples from non-transformed plants. Typical mRNA levels from fruit collected from three of these plants at breaker and three days post-breaker stages of development showed a fragment
hybridising at the expected size of the transgene message (Fig. 4). A total of 25 plants arising from independent transformation events using the p2ALX antisense construct were analysed. Four of these transformants, which by Southern blot analysis were shown to contain the transgene (Fig. 5), exhibited significant suppression of the targeted TomloxA and TomloxB endogenous message (Fig. 6). The four transgenic plants, transformed with the p2ALX construct, were initially selected by their ability to grow on kanamycin-containing media and subsequently shown to contain the intact transgene by Southern blot analysis (Fig. 5). Genomic DNA was digested with the restriction enzymes KpnI/BamHI, which cut within the inserted DNA and released a hybridising fragment of 5.2 Kb. The hybridising fragment was detected in the four independent p2ALX transformants but was not found in wild-type non-transformed plants (Fig. 5).

The four plants transformed with the p2ALX construct and showing suppression of the endogenous TomloxA and TomloxB mRNAs were
Table 1

Measurement of crude lipoxygenase (LOX) activity (μmol O₂/min per g FW), amount of protein (mg/g FW) and specific activity (μmol O₂/min per mg protein) from fruit samples of the four p2ALX antisense plants exhibiting suppression of the endogenous LOX message

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>LOX activity</th>
<th>Protein content</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (n = 3)</td>
<td>20.03 ± 0.03</td>
<td>1.18 ± 0.27</td>
<td>0.60 ± 0.11</td>
</tr>
<tr>
<td>2A11/LOX1.1O1 (n = 4)</td>
<td>20.03 ± 0.03</td>
<td>n.d.</td>
<td>0.83 ± 0.15</td>
</tr>
<tr>
<td>2A11/LOX1.1K1 (n = 4)</td>
<td>20.1</td>
<td>0.21 ± 0.03</td>
<td>0.56 ± 0.05</td>
</tr>
<tr>
<td>2A11/LOX1.1E1 (n = 4)</td>
<td>19.4 ± 0.65</td>
<td>n.d.</td>
<td>0.91 ± 0.18</td>
</tr>
<tr>
<td>2A11/LOX1.1H2 (n = 4)</td>
<td>12.7</td>
<td>n.d.</td>
<td>0.38 ± 0.06</td>
</tr>
</tbody>
</table>

*As controls fruit were harvested and measured from non-transformed wild-type plants. Fruit were collected at 6 days post-breaker. (n.d., no detectable enzyme activity).

LOX1.1 O1, LOX1.1 K1, LOX1.2 E1 and LOX1.1 H2 (Fig. 6). The mRNA expression results are expressed as a percentage of the maximum radioactivity (cpm) achieved for each of the individual probes. In the case of TomloxA and TomloxB the level of suppression of the endogenous LOX mRNA did not follow the same trend in fruit from the four transformed plants. For example, fruit from the plant LOX1.1 O1 showed the greatest suppression of TomloxA mRNA (4 and 2% at breaker and 3 days post-breaker, respectively) compared with wild-type levels (Fig. 6). However, when TomloxB was used as a probe (Fig. 6), the greatest level of suppression appeared to be in the fruit samples from plant LOX1.2 E1 (5.4 and 7.5% of wild-type levels at breaker and 3 days post-breaker, respectively).

In contrast, when TomloxC was used as a probe (Fig. 6), the pattern of expression showed a clearly different trend to those of TomloxA and TomloxB. Instead of maximum expression occurring in wild-type fruit, maximum expression of TomloxC occurred in breaker and 3 days post-breaker fruit in samples from the plant designated LOX1.1 O1, which had previously shown maximum suppression when probed with TomloxA (Fig. 6). Furthermore, whereas in wild-type fruit probed with TomloxC maximum expression was detected at 3 days post-breaker, maximal expression in LOX1.1 O1 transformed fruit was detected at the breaker stage of development (Fig. 6). Similarly, fruit from the plant designated LOX1.1 H2 exhibited this switch in maximal expression from 3 days post-breaker fruit to breaker fruit (Fig. 6). However, TomloxC expression only exceeded wild-type levels at the breaker stage of fruit development in this transformant (Fig. 6).

3.2. LOX enzyme assays

The mRNA expression results clearly indicated that fruit from several plants transformed with the p2ALX antisense construct exhibited major suppression of the endogenous TomloxA and TomloxB mRNAs. Fruit from all four transgenic plants that showed reduced mRNA levels were collected for LOX enzyme analysis at 6 days post-breaker. As controls, fruit samples were harvested from non-transformed wild-type plants.

LOX enzyme activity in fruit samples from plants LOX1.1 K1, LOX1.1 O1, LOX1.2 E1 and LOX1.1 H2 was either not detectable or extremely low compared with control samples (Table 1). Measurements of protein content of the crude LOX extracts (Bio-Rad protein assay) showed similar amounts of protein in extracts from control and transgenic fruit (no significant difference at P > 0.05). The reduction in enzyme activity in down-regulated fruit was therefore not due to inefficient extraction but due to successful suppression of LOX in the four suppressing plants compared with wild-type (P > 0.05) (Table 1).

3.3. Volatile analysis

The relative amounts of volatiles generated by controlled maceration of the fruit samples ((Z)-3-hexenal, (E)-2-hexenal and hexanal are formed
enzymically on maceration) together with others already present in fruit prior to maceration were measured (Table 2). Comparison of the relative amounts of the two volatile groups shows the natural variability in volatile content between individual fruit (see especially the variation in isobutylthiazole content). The volatile composition of the wild-type fruit shows a similar pattern.

Analysis of variance showed no significant differences between the relative amounts of hexanal and the combined (Z)-3-hexenal/(E)-2-hexenal composition of wild-type and the four transgenic suppressed fruit lines (Table 2). The sequential activity of LOX and hydroperoxide lyases on linolenic (18:3) acid gives rise to (Z)-3-hexenal and (E)-2-hexenal which isomerize, which is why their values are combined. Interestingly, the amounts of 1-penten-3-one were reduced in the four LOX suppressing lines compared with wild-type (Table 2).

4. Discussion

LOX have been proposed to play a role in many plant physiological processes, ranging from wound and stress responses (Heitz et al., 1997), to the synthesis of regulatory molecules (Bell et al., 1995). To date, few reports appear in the literature on gene silencing approaches to investigate the role of LOX genes and their enzyme products. Although transgenic tomatoes exhibiting suppression of TomloxB have been produced, reports on their biochemical characterisation are awaited (Kausch and Handa, 1997). Progress has been made in elucidating the role of the chloroplast-located LOX isozyme LOX2 in jasmonic acid (JA) accumulation (Bell et al., 1995). Analysis of transgenic A. thaliana plants revealed that gene silencing resulted in the reduction in levels of endogenous LOX2 mRNA, which was associated with the absence of wound-induced JA accumulation.

Initially, it was attempted to down-regulate LOX using antisense constructs driven by the 35S promoter. No transformants were recovered, perhaps because reduction in LOX is detrimental to plant regeneration. The 1.2 Kb antisense LOX construct p2ALX, driven by the fruit specific 2A11 promoter, generated tomato fruit with reduced levels of endogenous TomloxA and TomloxB mRNA (Fig. 6). In addition, LOX enzyme activity was either close to zero or not detectable in the four p2ALX antisense plants exhibiting suppression of the two endogenous LOX transcripts (Table 1). Antisense genes expressed in transgenic tomatoes have been used in previous studies to identify and down-regulate genes encoding enzymes involved in carotenoid biosynthesis, cell wall degradation and ethylene synthesis (Gray et al., 1992). Although the ripening specific PG promoter has been used in studies to manipulate alcohol dehydrogenase levels (Speirs et al., 1998), to the authors’ knowledge this is the first report of altering enzyme activity in tomato using a fruit-specific antisense approach. Furthermore,

Table 2
Peak areas for volatiles, standardised for weight and internal standard, analysed in fruit samples at 6 days post-breaker 

<table>
<thead>
<tr>
<th>Volatile</th>
<th>Wild-type (n = 3)</th>
<th>LOX1.1O1 (n = 4)</th>
<th>LOX1.1K1 (n = 2)</th>
<th>LOX1.2E1 (n = 3)</th>
<th>LOX1.1H2 (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Methyl-butanal</td>
<td>3.20 ± 1.18</td>
<td>3.92 ± 1.55</td>
<td>3.44 ± 1.99</td>
<td>5.45 ± 1.80</td>
<td>3.56 ± 0.20</td>
</tr>
<tr>
<td>1-Penten-3-one</td>
<td>23.20 ± 5.91</td>
<td>4.71 ± 2.05</td>
<td>12.35 ± 3.72</td>
<td>9.97 ± 2.24</td>
<td>11.46 ± 0.64</td>
</tr>
<tr>
<td>3-Methyl-butanol</td>
<td>2.91 ± 1.42</td>
<td>2.66 ± 1.95</td>
<td>1.35 ± 0.87</td>
<td>5.07 ± 5.01</td>
<td>3.78 ± 0.27</td>
</tr>
<tr>
<td>Hexanal</td>
<td>16.78 ± 2.94</td>
<td>13.02 ± 5.55</td>
<td>13.48 ± 1.81</td>
<td>22.82 ± 9.72</td>
<td>17.93 ± 1.78</td>
</tr>
<tr>
<td>2-Methylbutanal</td>
<td>3.54 ± 2.20</td>
<td>2.46 ± 0.96</td>
<td>3.41 ± 3.66</td>
<td>3.68 ± 3.28</td>
<td>2.17 ± 0.21</td>
</tr>
<tr>
<td>2-Isobutylthiazole</td>
<td>4.47 ± 5.38</td>
<td>4.45 ± 4.34</td>
<td>3.58 ± 2.88</td>
<td>0.88 ± 0.81</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>(Z)+(E) hexenal</td>
<td>40.57 ± 8.22</td>
<td>33.43 ± 15.00</td>
<td>28.81 ± 0.63</td>
<td>57.75 ± 13.46</td>
<td>43.47 ± 3.12</td>
</tr>
</tbody>
</table>

* Fruit samples were harvested from the four p2ALX antisense plants exhibiting suppression of the endogenous lipoxygenase (LOX) message. As controls, fruit were harvested and measured from non-transformed wild-type plants: n = number of fruit analysed.
the findings demonstrate that in this particular case the lack of a terminator in the p2ALX antisense construct did not prove to be deleterious in targeting endogenous LOX transcripts for down-regulation.

Transgenic plants generated using the PG promoter and terminator with a ~400 bp LOX antisense insert showed little if any suppression of the endogenous message (Fig. 4), although the transgene message did accumulate as a fragment of the correct size. The lack of detectable suppression of the endogenous message may have been due to the developmental stage at which the PG promoter is switched on (Nicholass et al., 1995), and the late onset of antisense expression compared with the earlier onset of expression of the endogenous LOX genes (Fig. 3). Alternatively, the relatively short antisense sequence used in this construct (~400 bp) may be a reason for the lack of significant suppression in these plants. However, this is considered unlikely, as fragments of 150 bp have been shown to be effective in antisense experiments with tomato PG (Grierson and Smith, unpublished), and a 41 bp fragment has been shown to be effective in down-regulating the GUS gene in transgenic tobacco (Cannon et al., 1990).

Studies aimed specifically at manipulating tomato flavour by the production of genetically modified plants have been limited. One study involved expression of the yeast Δ-9 desaturase gene, resulting in reported changes in fatty acid and fatty acid-derived flavour compounds (Wang et al., 1996). In plants transformed with the p2ALX antisense construct, if LOX was responsible for the generation of flavour volatiles, the suppression of the endogenous TomloxA and TomloxB message (Fig. 6), plus the absence of measurable LOX enzyme activity, should have resulted in significant reductions in C6 volatile production, specifically the C6 aldehydes hexanal and hexenal. There was no trace of the C6 alcohols suggesting that the initial stages of the pathway (LOX, lyase and isomerase) are functioning but that the final stage (alcohol dehydrogenase) was not significant under these conditions of maceration and analysis. Aroma volatile analysis of fruit samples from the four antisense LOX-suppressing plants indicated no statistically significant effects compared with wild-type (Table 2). However, the amount of 1-penten-3-one was reduced in the LOX-suppressing plants. Although 1-penten-3-one is not recognised as a common lipid oxidation volatile (Frankel, 1998), it is possible that it is formed from some of the known C5 compounds which have been found in auto-oxidation or photo-oxidation of lipids. It is possible that an additional LOX isoform exists possessing a C5-forming capability which has been suppressed in the current study.

The fact that (E)-2-hexenal has the largest peak area (Table 2) does not necessarily mean that there is more of this compound than the others. Volatile amounts are given in integrated peak areas for a characteristic ion. This allows direct comparison of the amounts of a single compound in the control and down-regulated fruits. However, the relationship between the characteristic ion peak and the actual amounts of compounds (expressed as μg/g) will differ for individual compounds.

Highly variable levels of volatiles measured between individual fruit samples have been encountered in earlier studies (Kazeniac and Hall, 1970), and this did not aid the interpretation of the data in the present experiments. The magnitude of the inherent variation could mask effects of down-regulation of the targeted LOX. It seems probable, however, that either extremely low levels of LOX enzyme activity are sufficient for volatile generation or the LOX isozymes targeted are not responsible for generation of these volatiles.

In the present experiments TomloxA and TomloxB transcripts were successfully down-regulated. TomloxC mRNA, however, was not reduced and showed increased expression in some transformed lines compared with wild-type. The predicted changes in the aroma volatiles (Z)-3-hexenal, (E)-2-hexenal and hexanal were not observed, although unexplained changes in 1-penten-3-one were detected. Considering the extent of suppression of the targeted endogenous TomloxA, and the closely related TomloxB transcript, particularly in some lines, and the fact that LOX enzyme activity was not detectable, these results may appear surprising. However, these findings also ex-
emphasize the great versatility of plant metabolism. In successfully down-regulating Tomlox_A and Tomlox_B expression, a switch to an alternative LOX activity, that of Tomlox_C appeared to occur. Furthermore, the developmental stage of maximal expression of Tomlox_C appeared to change from 3 days post-breaker to breaker when other LOX isoforms were reduced. It is possible that Tomlox_C could encode the key LOX enzyme involved in C6 volatile generation (Fig. 6). This would not be unprecedented, in view of findings from soybean, (Moreira et al., 1993), where the absence of LOX isozymes 2 and 3 in specific cultivars resulted in significant reductions in hexanal production, whereas, the absence of LOX isozyme 1 had little if any effect on hexenal levels. The N-terminal extension of Tomlox_C has been suggested to serve as a chloroplast transit peptide (Heitz et al., 1997), indicating it may function in a different cell compartment from the other LOX isozymes. During the course of fruit ripening thylakoid membrane breakdown accompanies the transition of chloroplasts to chromoplasts. The release of membrane lipids in this process and the possible involvement of a specific LOX, possibly Tomlox_C or Tomlox_D, which may also be chloroplast targeted (Heitz et al., 1997), could contribute significantly to the generation of the C6 aroma volatiles. The findings indicate either that LOX enzyme activity is not rate limiting in the generation of aroma volatiles or that a specific LOX activity not down-regulated in the present experiments participates in this process.

Acknowledgements

The authors wish to thank Dr Rod Casey for assistance in the initial library screening work and Andrew Wallace and Dr Rupert Fray for supplying the LOX3 (Tomlox_C) partial length cDNA clone.

References

wild-type and mutant tomato fruit development. Plant Physiol. 113, 1041–1050.