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^{13}C-labelling patterns of green leaf volatiles indicating different dynamics of precursors in *Brassica* leaves

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Abstract

To investigate the dynamics of precursor compounds of green leaf volatiles (GLV)s and other biogenic compounds released by mechanically damaged *Brassica oleracea* leaves, plants were exposed for two consecutive 16 h light phases to highly enriched $^{13}CO_2$. Analysis by GC-MS indicated (1) biogenic compounds released upon wounding, (2) a different labelling pattern between and (3) within compounds, and (4) evidence for spatial heterogeneity of the precursor pool extrapolated from points (1)–(3).

First, GLVs comprised C_5 and C_6 molecules, with the GLV pentenyl acetate being reported here for the first time from higher plants.

Second, the labelling pattern found in most GLVs indicates a low turnover of the precursor α-linolenic acid. Moderate labelling of dimethyldisulphide indicates a connection to an active plastidic methyl pool closely connected to CO_2 fixation, and very weak labelling of terpenes indicates a constitutive monoterpene pool.

Third, not all GLVs exhibit similarly strong labelling patterns (hexenyl acetate vs. hexyl acetate), indicating different precursors. As the labelling patterns of alcohol and acetate moieties in the esters differ, with only the former being strongly labelled, the precursor of the acetate moiety, acetyl-CoA, is likely to derive from a different cellular pool to that used in chloroplastic fatty acid synthesis, or was rapidly synthesised after the end of labelling.

Fourth, the exceptionally high relative abundance of labelled GLV and the low concentration of unlabelled molecules are likely to occur because recently synthesized α-linolenic acid is bound in lipids that are organised in distinct areas, or are chemically different from the older lipids. They must be preferentially used as precursors.

Keywords: *Brassica oleracea* L. var. *gemmifera*; Brussels sprout; Green leaf volatiles; Hexenyl acetate; Dimethyldisulphide; Lipoxygenase; Stable isotope analysis; Biosynthesis; Compartmentation

1. Introduction

Plants have been shown to emit volatile defence compounds in response to damage by herbivores, pathogens and mechanical forces. In *Brassica* plants, mechanical damage induces release of green leaf volatiles (GLV)s and terpenes (Connor et al., 2007; Scascighini et al., 2005).

GLVs are important defensive compounds in a number of plant species, and have been shown to act by repelling herbivores (Kessler and Baldwin, 2001), affecting their fecundity (Hildebrand et al., 1993), and by intoxicating microbes (Croft et al., 1993). They can also contribute to indirect defence by attracting natural enemies of herbivores (Mattiacci et al., 2001) as well as by warning neighbouring plants of possible attack (Engelberth et al., 2004). Undamaged leaf tissues contain and release only low quantities of GLVs but upon disruption by herbivores, microbes or mechanical damage rapid synthesis occurs (Matsui et al., 2000, 2006). GLVs are synthesised via the LOX (lipoxygenase) pathway from C_{18} polyunsaturated fatty acids including linoleic acid and linolenic acids (Dudareva et al., 2006;

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Matsui, 2006). The C_{18} acids are cleaved to C_{12} and C_6 compounds by hydroperoxide lyases (Engelberth et al., 2004; Hatanaka et al., 1987). Additionally, C_5 compounds have been observed as minor GLV constituents (Salch et al., 1995). The first C_6 GLV compound synthesised by the LOX/lyase pathway is 3*Z*-hexenal which is formed within seconds upon tissue disruption (Matsui et al., 2000) and is then converted to other GLVs such as 2*E*-hexenal (leaf aldehyde), 3*Z*-hexenol (leaf alcohol) and 3*Z*-hexenyl acetate (leaf ester) (Hatanaka et al., 1987; Shiojiri et al., 2006). 3*Z*-Hexenyl acetate is formed from a reaction between 3*Z*-hexenol and acetyl-CoA that is catalysed by an acyltransferase (D'Auria et al., 2007).

Paré and Tumlinson (1998) reported labelling experiments for 3*Z*-hexenyl acetate in cotton plants. The authors found that following labelling with $^{13}CO_2$, the alcohol moiety of 3*Z*-hexenyl acetate was unlabelled, while the acetate moiety showed strong labelling, and concluded that α-linolenic acid, the precursor of the alcohol moiety, is obtained from storage products. Converse to the weak labelling of the GLVs, the authors found that the rapid loss of labelling in the terpene fraction after pulse labelling indicated a rapid turnover of these compounds (Paré and Tumlinson, 1997). Terpenoids have been shown to be very important in tritrophic insect-plant interactions (Dicke and Sabelis, 1988). Terpenoids induced by feeding insects include monoterpenes (C_{10}) (Hern and Dorn, 2002), sesquiterpenes (C_{15}) (Hern and Dorn, 1999), homoterpenes (C_{11} and C_{16}) (Turlings et al., 1990), diterpenes (C_{20}) (Miller et al., 2005) and triterpenes (C_{30}) (Dutton et al., 2002). Such induced compounds guide natural enemies to the site of damage (Dutton et al., 2002). Volatile terpenoids are produced by the cytosolic mevalonate pathway or via the DOXP/MEP (1-deoxy-D-xylulose-5-phosphate/2-C-methyl-D-erythritol 4-phosphate) pathway in the plastids (Arimura et al., 2005). Mevalonate derived terpenoids are synthesized in the cytoplasm from acetyl-CoA, that is converted to mevalonic acid, which in turn is used to form isopentyl pyrophosphate (IPP), the precursor of all terpenes. IPP is also an intermediate of biosynthesis via the DOXP/MEP pathway.

Although CO_2 fixed in the stromal compartment of the chloroplasts (Heldt et al., 1977) is the ultimate source of carbon for fatty acid synthesis (Liedvogel, 1986), acetyl-CoA can be regarded as the more direct precursor (Harwood, 1988). It is interesting to note that up to 75% of the total cellular acetyl-CoA is found in plastids (Post-Beittenmiller et al., 1992; Tumaney et al., 2004). In the plastids, acetyl-CoA is carboxylated to form malonyl-CoA which is a substrate for *de novo* fatty acid synthesis (Masterson et al., 1990; Post-Beittenmiller et al., 1992), but the question is still open as to whether the precursors of acetyl-CoA are imported from the cytosol into the chloroplasts or whether CO_2 fixation products serve solely as precursors (Rawsthorne, 2002).

The principal objective of the current investigation was to gain insight into the biogenic origin of the liberated volatile compounds of mechanically damaged *Brassica oleracea* plants. To extrapolate the dynamics of the precursors of GLVs and other volatiles, analysis of the molecular ^{13}C labelling patterns was conducted by GC-MS following exposure to highly enriched $^{13}CO_2$.

2. Results

Following exposure to a highly $^{13}CO_2$ enriched atmosphere for two consecutive 16 h light phases, one leaf of *Brassica* plants was damaged with a hole punch and volatiles emitted were collected for 2 h. A high number of volatile compounds emitted from punctured leaves were detected by combined gas chromatography – low resolution EI mass spectrometry (Table 1). Biogenic compounds included GLVs, monoterpenes, 2,4-hexadienal and dimethyldisulphide (Table 1). 3*Z*-Hexenyl acetate and 3*Z*-hexenol were the dominating compounds of the bouquet.

Strong labelling was observed in nearly all fatty acid derived compounds as is exemplified in Figs. 1 and 2. Labelling was moderate in dimethyldisulphide, and very weak in the monoterpene fraction and in 2,4-hexadienal. Statistical analysis confirms the low labelling of the monoterpenes limonene and myrcene (Fig. 2), while no statistical evidence supports the low percentage of labelling shown in α-pinene, which is likely to result from small sample size ($n = 2$; i.e., maximal labelling detected in 2 of 7 samples). In 2,4-hexadienal the level of significance was just missed ($P = 0.1$).

Xylene exhibited negligible labelling, reflecting the natural abundance of ^{13}C (Fig. 2); this compound represents an example of one of the many anthropogenic contaminants that were present in the samples.

Of particular interest was the labelling pattern of the esters, which combine carbon atoms derived from acetate, and alcohol moieties that are cleavage products of polyunsaturated fatty acids. To determine the position and abundance of the ^{13}C label in the two moieties of the esters, mass shifts of indicator fragment ions were applied (Table 1). The fragment ions *m/z* 82 and *m/z* 84, and the oxygen containing fragment ion *m/z* 86 were used to determine the labelling of the alcohol moieties of 3*Z*-hexenyl acetate, hexyl acetate and 2*Z*-pentenyl acetate, respectively. The empirical formula of the fragment ion *m/z* 86 $[C_5H_{10}O]^+$ was confirmed by a labelled compound that we synthesized. The fragment ions *m/z* 43 of the compounds served as indicator ion for the acetate moieties of the alkyl acetate and alkenyl acetates. Synthesized ^{13}C acetate labelled esters allowed correction for the small contribution of isobaric hydrocarbon fragment ions to this ion.

For some other compounds (monoterpenes and 2,4-hexadienal), the determination of labelling was restricted to a reduced number of carbons of the molecules because suitable ions with a sufficient abundance of all carbons were lacking for the applied mass spectrometric conditions. In addition, the positions of the ^{13}C labelling in the carbon chains could not be determined.

Table 1
Empirical formulae of fragment ions used as indicator ions and intensities (mean peak area × 10^3 ± SE) of the biogenic products from mechanically damaged leaves of *Brassica oleracea*

Compound	Fragment composition	Moiety	*n*	Mean peak area	^{12}C- Acetate *m*/*z*	^{13}C- Acetate *m*/*z*
2*Z*-Pentenol	$[C3H5O]^+$		4	137 ± 18	57	
2*Z*-Pentenyl acetate	$[C5H10O]^+$	Alcohol	6	312 ± 36	86	86
2*Z*-Pentenyl acetate	$[C3H7]^+$	Alcohol			43	43
2*Z*-Pentenyl acetate	$[CH3CO]^+$	Acetate			43	45
2*E*-Hexenal	$[C5H7O]^+$		7	169 ± 32	83	
3*Z*-Hexenol	$[C6H10]^+$		6	3522 ± 1663	82	
2,4-Hexadienal	$[C5H5O]^+$		4	365 ± 180	81	
3*Z*-Hexenyl acetate	$[C6H10]^+$	Alcohol	6	8520 ± 2105	82	82
3*Z*-Hexenyl acetate	$[C3H7]^+$	Alcohol			43	43
3*Z*-Hexenyl acetate	$[CH3CO]^+$	Acetate			43	45
Hexyl acetate	$[C6H12]^+$	Alcohol	3	321 ± 97	84	84
Hexyl acetate	$[C3H7]^+$	Alcohol			43	43
Hexyl acetate	$[CH3CO]^+$	Acetate			43	45
Dimethyldisulphide	$[C2H6S2]^+$		4	137 ± 19	94	
α-Pinene	$[C7H9]^+$		7	711 ± 78	93	
Limonene	$[C7H9]^+$		7	1964 ± 334	93	
Myrcene	$[C7H9]^+$		6	1619 ± 609	93	
Xylene	$[C7H7]^+$		7	3453 ± 641	91	

The mass shift of indicator ions when labelled with $^{13}C_2$-acetate are provided. The number of samples in which the compound was detected (*n*) is from a maximum of 7.

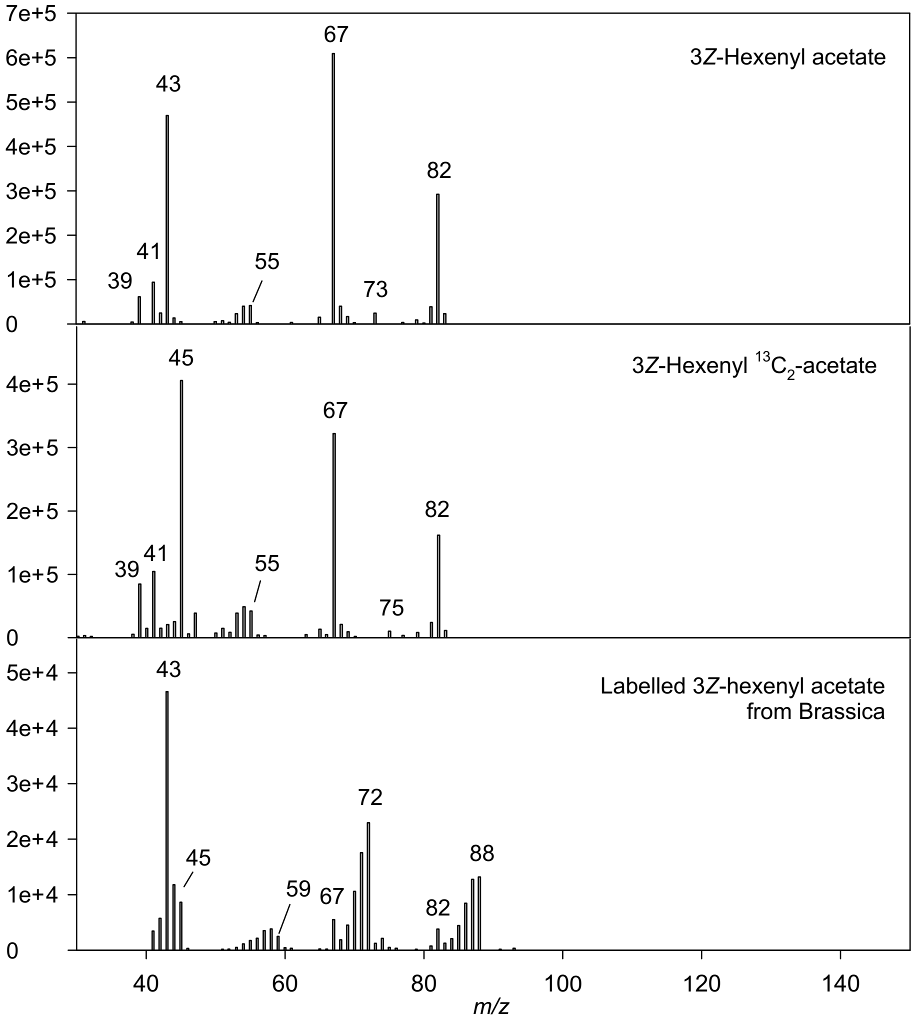

Fig. 1. Mass fragmentation patterns of 3*Z*-hexenyl acetate (natural abundance of carbon isotopes), 3*Z*-hexenyl $^{13}C_2$-acetate and ^{13}C labelled 3*Z*-hexenyl acetate detected from mechanically damaged *Brassica* plants exposed to highly enriched $^{13}CO_2$ for two consecutive 16 h light phases.

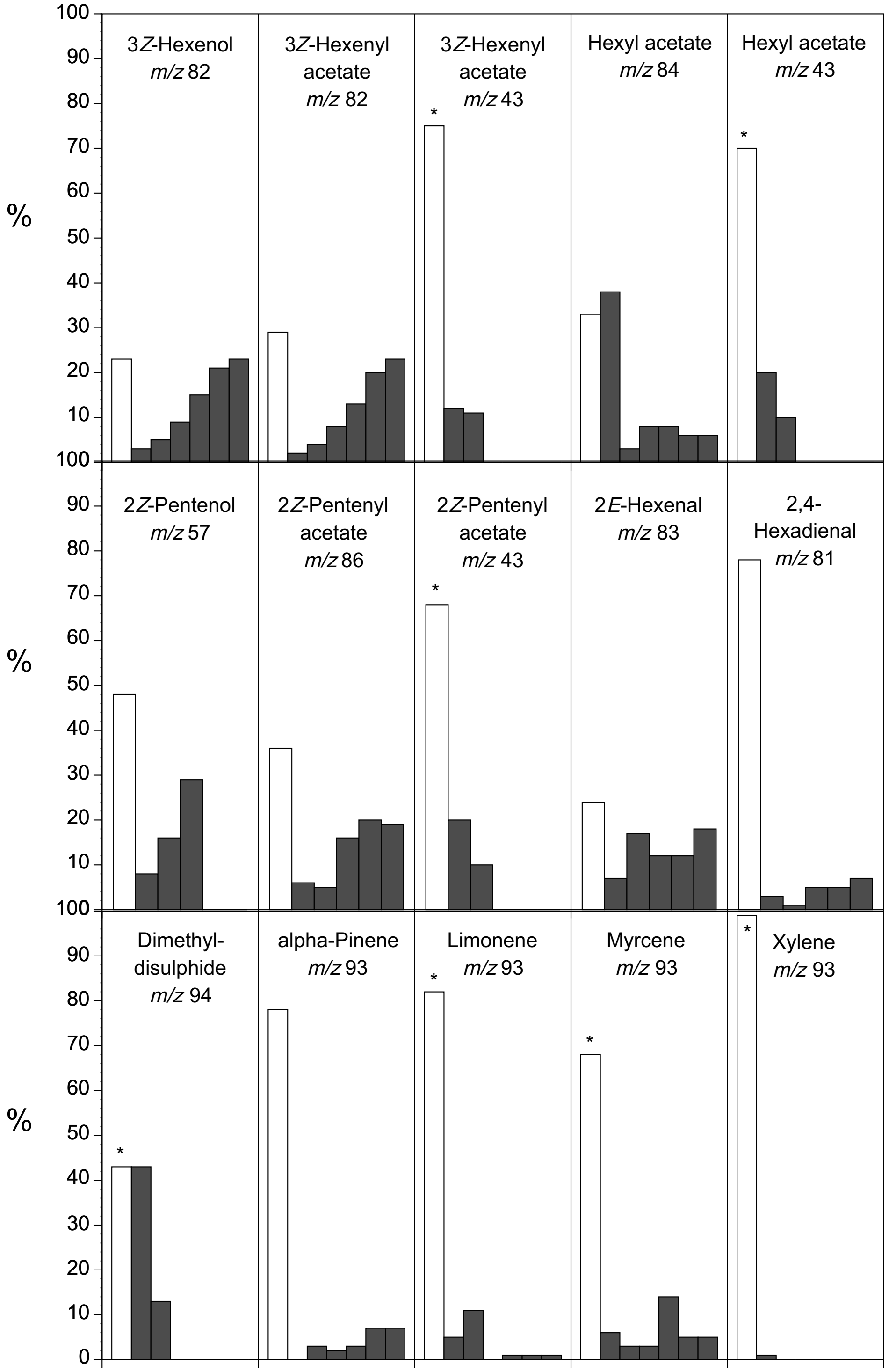

Fig. 2. *Y*-axis: Relative abundance pattern of labelling in volatile compounds (in%). *X*-axis: White columns represent fragment ions with no ^{13}C incorporation into the molecule. Dark columns represent fragment ions with one to seven ^{12}C atoms of the molecule replaced by ^{13}C. * Represents a significant difference between the non-labelled molecular species (white column) and the maximally labelled molecular species (right-most dark column) ($P < 0.05$ level, Mann-Whitney U-test).

Remarkably, 70% of the C_6-GLV molecules detected in the headspace of mechanically damaged *Brassica* plants contained at least one ^{13}C atom (Fig. 2). The once-^{13}C-labelled molecules exhibited very low relative abundances, but these abundances increased with the number of ^{13}C atoms per molecule. The fully labelled molecular species (with maximum replacement of ^{12}C atoms by ^{13}C atoms) were more abundant than those with fewer replacements of ^{12}C atoms by ^{13}C atoms. This also holds for 2*Z*-pentenol and its acetate ester. However, to calculate the labelling of the whole carbon skeleton of 2*Z*-pentenol, a suitable fragment ion with sufficient abundance was not available and the fragment ion *m*/*z* 57 $[C_3H_5O]^+$ that is generated by the loss of the terminal carbons 4 and 5 had to be used (Fig. 2). Since the two missing carbons were not included in the calculation of percentage abundance, this yielded an artificially high proportion of unlabelled molecules. Assuming a continuous increase of relative abundance with the labelling number, the unlabelled molecules are in the same range as in 3*Z*-hexenol and 3*Z*-hexenyl acetate, when the missing carbons are theoretically added. This is not the case for hexyl acetate since the alcohol moiety is only weakly labelled.

The acetate moieties of the esters differed essentially in the relative abundance of labelled species. All acetate moieties exhibited low labelling, and the once labelled species were much more abundant than the twice labelled.

3. Discussion

3.1. Brassica volatiles

Among the numerous compounds identified in the headspace of mechanically damaged *Brassica* plants, GLVs quantitatively prevail. While GLVs are usually defined as saturated and unsaturated C_6 alcohols, aldehydes and esters (e.g. Paré and Tumlinson, 1996), this study conclusively shows that C_5 compounds can be constituents of the GLVs as well. In fact, one of these C_5 GLVs has not been previously described (2*Z*-pentenyl acetate), and one has rarely been reported from higher plants (2*Z*-pentenol by Salch and coauthors 1995). In addition, several monoterpenes and dimethyldisulphide were detected in accordance with previous publications (Blaakmeer et al., 1994; McEwan and Macfarlane Smith, 1998; Scascighini et al., 2005).

3.2. Labelling differences between compounds

The results of our study reveal some new dynamics of the precursors of GLVs and other biogenic compounds from *Brassica* plants. All GLVs were strongly labelled by $^{13}CO_2$ and exhibited labelling patterns that differed in both the number of labelled C atoms per molecule and in the abundance of these molecular species.

The relative abundances of molecules with different numbers of ^{13}C atoms were very similar in the different GLV compounds, irrespective of whether the chain length was C_5 or C_6. The highest relative abundance was found in the majority of cases for the fully labelled molecules and a linear decrease of relative abundance was observed concomitant with decreasing numbers of ^{13}C label in the molecules (Fig. 2).

The appearance of the series of one- to five/six-times labelled C_5 and C_6 compounds indicates that the labelled fatty acid precursors had chain lengths of less than C_{18} at the beginning of exposure to $^{13}CO_2$. The number of labelled C atoms reflects the chain elongation. This direct relationship can be postulated since the experimental atmosphere contained highly enriched $^{13}CO_2$. For a step-wise increase of labelling, there are two requirements: Firstly, a small pool size of the precursor molecules because only a limited labelling period was provided and secondly, a negligible turnover of the precursors within the labelling period.

If a rapid turnover of α-linolenic acid would have taken place, then the maximum percentage labelling in its products should have been found for once labelled alcohols and alcohol moieties of the esters with a decreasing amount for the twice and further replacements. However, in our experiments the opposite was found. In 3*Z*-hexenol, 2*Z*-pentenol, 2*E*-hexenal, and the alcohol moieties of 2*Z*-pentenyl and 3*Z*-hexenyl acetate, an increasing percentage concomitant with an increasing number of ^{13}C atoms per molecule was observed.

The difference in the labelling pattern of 2,4-hexadienal and the hexyl moiety to the other GLVs strongly suggests that the precursors of these compounds are not identical. This difference may be due to molecular separation or spatial separation (cytoplasmic membranes) of the precursors of 2,4-hexadienal and the hexyl moiety from that of the other GLVs.

In 2,4-hexadienal, a polyunsaturated aldehyde, an unprecedented number of molecules were unlabelled. Labelling of a similarly low range was also observed in the alcohol moiety of hexyl acetate. It is possible that the unexpectedly high labelling of the once labelled C_6 moiety of this compound may be an artefact and could be explained by the presence of an unknown compound at the same retention time.

Dimethyldisulphide exhibited a moderate level of labelling indicating that its precursors are members of an active metabolism that is closely connected to CO_2 fixation products. The precursor of this compound is only slightly diluted by carbons derived from storage products. Several reactions have been described for the formation of methanethiol (Schmidt et al., 1985; Schwenn et al., 1983), which is most likely the immediate precursor of dimethyldisulphide (Engels et al., 2000). The labelling of dimethyldisulphide indicates a close connection to an active chloroplastidic methyl pool. Oxidation mediated by active oxygen species that are formed during cell damage readily affords dimethyldisulphide from methanethiol (Engels et al., 2000). This chemically mediated oxidation does not appear to be the sole reaction leading to the disulphide, as the ^{13}C label was not equally distributed across both carbons.

α-Pinene and myrcene exhibited a labelling pattern that indicates a minor contribution of newly formed molecules to the non-labelled constitutive monoterpene pool. The completely labelled molecular species showed the highest relative abundance, and abundances decreased linearly with decreasing numbers of ^{13}C per molecule. This pattern would be expected when no induction or activation as shown for other terpenoids (Paré and Tumlinson, 1997) takes place.

3.3. Labelling differences within compounds

A previous study described ^{13}C labelling of plant derived 3*Z*-hexenyl acetate in cotton (Paré and Tumlinson, 1998). Preferential acetate labelling with no labelling of the 3*Z*-hexenyl moiety was reported from this plant when systemically induced by caterpillar feeding (Paré and Tumlinson, 1998). In our study with *Brassica* plants that were mechanically damaged, the majority of the acetate moieties in the esters were unlabelled, while the opposite was found for the alcohol moieties that exhibited an increase in label with the number of replaced ^{12}C carbon atoms.

There are several differences between the present study and the study by Paré and Tumlinson (1998) including plant species, $^{13}CO_2$ concentration (current study: 900 ppm vs. their study: 360 ppm), labelling period (32 h total vs. 9 h total), air-flow (3.6 l/h vs. 30 l/h), as well as induction and sampling. We believe that the two last aspects may account for the stark differences in the labelling patterns observed. First, we analysed emissions from mechanically damaged leaves on the plant, while Paré and Tumlinson (1998) analysed emissions from undamaged leaves on plants that were systemically induced by caterpillar feeding. Second, we analysed the emission of volatiles after the $^{13}CO_2$ fixation period, while the other authors collected volatiles during $^{13}CO_2$ fixation.

The different pattern of alcohol and acetate moiety labelling can be explained by the respective turnover times of these molecules. In our experiment the 10-h time interval between the end of $^{13}CO_2$ labelling and the beginning of volatile collection seems to be long enough to replace most of the $^{13}CO_2$ from the acetate pool that was used in the formation of GLV esters. The other study (Paré and Tumlinson, 1998) showed that despite the increased release of volatiles from systemically induced leaves, only the acetate moieties of esters contained ^{13}C label. The C_6 compounds were not labelled although the labelling time should have been long enough.

The lower labelling exhibited by the acetate moieties compared with the alcohol moieties of the GLV esters suggests either dilution by carbon from compounds synthesized prior to the onset of the labelling experiment, or that the acetate detected was synthesised after the end of the labelling period. The acetyl-CoA pool contributing to ester formation was clearly different (spatial or temporal) from the acetyl-CoA pool feeding into the fatty acid precursors of the GLVs. The maximum label was found for the once-labelled and was less for the twice-labelled acetate moiety indicating that a rapid turnover of the precursors that feed into the pool of acetyl-CoA had taken place and caused the observed labelling of the carbons. Alternatively, another acetyl CoA pool may have been utilised that received acetate from weakly labelled storage material. It is important to note that as many as five different pools of acetyl-CoA have been demonstrated in eukaryotic cells (Fatland et al., 2005).

3.4. Extrapolations regarding spatial heterogeneity

α-Linolenic acid is most likely the precursor of the GLVs that are formed via a lipoxygenase mediated formation of 13(*S*)-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid and subsequent cleavage by lyases (Hatanaka et al., 1987; Salch et al., 1995). Our finding that unlabelled compound species were always detected at only a low level of around 20%, and the once or twice labelled species were even lower as opposed to the high abundance of multiple and totally labelled molecules, clearly indicates that the fatty acid precursors of these volatiles exhibited no or a very low turnover within the experimental period. This result is in line with the report that the specific radioactivity of α-linolenic acid in monogalactosyldiglycerides was retained over a long period (Roughan, 1970). It seems likely that the precursors originated from a small pool because if all of the fatty acids of the cell were equally utilised, a lower abundance of labelling for the fully labelled molecular species should have been observed.

To explain the low turnover of precursor fatty acids as indicated by the GLV labelling, a spatial separation or structural difference of the newly synthesised lipids should be considered as an option. The labelling time of 2 × 16 h was not sufficient to label all α-linolenic acid molecules in the leaves of *Brassica* plants, reflected by the low percentage labelling of 2,4-hexadienal. Development of a leaf to the size that it had in our experiment lasted much longer (2–3 weeks) than the relatively short period of exposure to $^{13}CO_2$ under our experimental conditions. It was therefore surprising that unlabelled compound species of GLVs were found in such low relative abundances. Conversely, the exceptionally high relative abundance of labelled GLV molecules would become understandable when recently synthesized α-linolenic acid is bound in lipids that are more easily accessible to lipases for cleavage than the majority of lipids synthesised previously. Obviously, these recently synthesised lipids are initially stored in distinct areas or exhibit chemical structures that are more accessible to the lipase compared to lipids that have been present for a longer time.

Several hydroperoxide lyases or specificities are necessary for the formation of the observed GLVs and their related compounds. A monooxygenase that cleaves between C_{13} and C_{14} of the fatty acid derivative, which would explain the formation of 2*Z*-pentenol, has been described (Kondo et al., 1995; Salch et al., 1995). The well characterized heterolytic hydroperoxide lyase would be responsible for the cleavage between C_{12} and C_{13} liberating a C_6 volatile (Feussner and Wasternack, 2002), and another lyases that are most likely positioned outside of the chloroplasts, but which are as yet not characterised, would liberate 2,4-hexadienal and hexanal (a precursor of hexyl acetate). The monounsaturated aldehydes, as opposed to the conjugated dienals, are readily reduced by an alcohol dehydrogenase. This reaction stabilizes the position of the double bond at position three in the less stable *cis*-configuration. The alcohols react in a nucleophilic acyl substitution reaction with acetyl-CoA catalysed by an acyltransferase (D'Auria et al., 2007). A compartmentation of these reactions is very likely to occur. Oxygenation and the cleavage reaction most probably take place in the chloroplasts (Feussner and Wasternack, 2002), while subsequent reactions at the base of the labelling pattern might occur in the cytoplasm (D'Auria et al., 2002).

4. Experimental

4.1. Plant material

Brussels sprouts (*B. oleracea* L. var. *gemmifera*) were reared as described previously (Scascighini et al., 2005). Plants used in experiments were 5 weeks old with 4–5 fully developed leaves and were similar in size, appearance and leaf number.

4.2. $^{13}CO_2$ labelling

Five-week old plants kept under 16:8 h L:D conditions were placed into an airtight Plexiglas box (350 mm × 450 mm × 350 mm, 20 °C, 1100 lux, 70% relative humidity) in groups of six and were provided with a $^{13}CO_2$ atmosphere (900–960 ppm CO_2 was measured at the beginning of each photosynthetic period, 150–250 ppm at the end) (99% $^{13}CO_2$; Cambridge Isotope Laboratories, Andover, MA, USA) for two consecutive days during the 16 h light periods. The Plexiglas box was flushed with $^{13}CO_2$ once per day at the beginning of the light phase (16 h) to ensure that plants took up $^{13}CO_2$. The box was then opened for the dark phase (8 h) to allow plants to respire under normal atmospheric conditions. On the third day, plants were removed from the box 2 h after the beginning of the light phase (10 h after the end of the second labelling period) and the uppermost fully developed leaf was damaged with 10 times 5 mm diameter holes made with a hole punch and volatiles were immediately collected for a 2 h period under light conditions.

4.3. Volatile collection

Volatiles were collected from the single leaf still attached to the *Brassica* plant. Collection started immediately following wounding and lasted for 2 h. The test leaf was isolated, using a Pyrex glass chamber described by Agelopoulos and coauthors (1999). Volatiles were collected in parallel from four plants onto steel traps containing 300 mg Tenax GR® (mesh size 80/100, Alltech Associates, Deerfeld, IL, USA), which were sealed with Pyrex glass wool at both ends. Charcoal filtered air entered the collection chamber through the inlet and was drawn out through the Tenax GR® trap at a rate of 340–360 ml/min for the duration of the collection. All collection was conducted in a climate room at 20 °C, 70% relative humidity and a 16:8 h light-dark regime.

4.4. Thermal desorption-GC-MS

Headspace volatiles were analysed using thermal desorption (Unity, Markes Int. Ltd™, Pontyclun, UK) connected to a GC-MS instrument (GC 6890 mass selective, MS detector 5973; Hewlett Packard, Atlanta, GA, USA). The thermal desorption details were as follows: Samples were desorbed at 300 °C from the Tenax GR trap with helium (99.96%) for 5 min (desorption flow 30 ml/min, split flow 10 ml/min). Volatiles were then transferred to a cold trap (−10 °C) which was filled with Carbopack B (Supelco, Park City, PA, USA) and Tenax TA (1:2). The cold trap was subsequently heated at a rate of 60 °C/min to 300 °C with a desorption time of 3 min. Volatiles were transferred via a fused silica transfer line (heated to 280 °C) to the GC-MS. The GC column used was a phenyl methyl siloxane coated capillary column (EC-5, 30 m, 0.25 mm i.d. and 0.25 µm film thickness, Alltech, Deerfield, IL, USA) fitted with a fused silica retention gap (HP1, 5 m, 0.25 mm i.d., HP Infochroma AG, Zug, Switzerland;). The initial temperature was 45 °C, which increased to 250 °C at a rate of 8 °C/min. A post run was conducted for 10 min at 300 °C, with helium used to flush out the system to remove any residual products from the previous sample. The desorption flow and column flow were kept at 30 ml/min and 1.5 ml/min, respectively, for all analyses.

4.5. Reference compounds

Reference compounds were obtained from Aldrich and Fluka (Buchs, Switzerland) and Roth (Karlsruhe, Germany). 2*Z*-Pentenol was synthesized from 2-pentin-1-ol. The *cis*-hydrogenation of 2-pentin-1-ol was readily accomplished by using dihydrogen and a Lindlar catalyst. 2*Z*-Pentenyl acetate was prepared by acetylation of the unsaturated alcohol with acetic anhydride. The EI-MS of the ester was *m*/*z* (%) 128 $[M]^+$ (0.8), 39 (15), 41 (22), 42 (8.3), 43 (100), 44 (2.6), 53 (12), 57 (17), 61 (5.3), 67 (36), 68 (37), 69 (10), 71 (4.2), 85 (7.4), 86 (19) and 99 (2.3). ^{13}C-Acetate labelled esters were synthesized from $^{13}C_2$-acetic acid (99 atom% ^{13}C, Sigma) and hexan-1-ol, 3*Z*-hexenol and 2*Z*-pentenol, respectively. Toluene sulfonic acid was used as the catalyst (Autorenkollektiv, 1988). The EI-MS for hexyl ^{13}C-acetate exhibited ions at *m*/*z* (%) 146 $[M]^+$ (<0.1), 39 (13), 41 (29), 42 (18), 43 (22), 44 (7.5), 45 (100), 55 (26), 56 (44), 61 (0.3), 63 (21), 69 (17), 73 (1.2), 75 (9.4) and 84 (22), for 3*Z*-hexenyl ^{13}C-acetate *m*/*z* (%) 144 $[M]^+$ (<0.1), 39 (21), 41 (26), 42 (3.4), 43 (4.6), 44 (6.4), 45 (100), 53 (9.5), 54 (12), 55 (10), 67 (79), and 82 (40), and for 2*Z*-pentenyl ^{13}C-acetate *m*/*z* (%) 130 $[M]^+$ (0.6), 39 (24), 41 (31), 42 (7.5), 43 (3.7), 44 (7.6), 45 (100), 53 (13), 57 (10), 67 (26), 68 (27), 85 (9.1), 86 (21), 99 (0.1) and 101 (2.3).

4.6. Indicator fragment ions

Indicator fragment ions were selected suitable for calculation of the differently labelled molecular species and molecular moieties (Table 1). Because the resolution of the mass spectrometer was not sufficient to differentiate between hydrocarbon and oxygen containing fragment ions, corrections were necessary. The fragment ion *m*/*z* 43 $[CH_3CO]^+$ characteristic of the acetate moiety of the esters contained minor amounts of the isobaric hydrocarbon $[C_3H_7]^+$. The contribution of the hydrocarbon ion to the total intensity of the ion *m*/*z* 43 was determined by application of synthesized ^{13}C-acetate labelled esters and found to be 3.6% for 2*Z*-pentenyl acetate, 4.4% for 3*Z*-hexenyl acetate and 18% for hexyl acetate.

4.7. Computer-aided data interpretation

A computer program was developed for data analysis and quantification of differently labelled molecular species and fragments. The program, written in Visual Basic 6, allows two kinds of simulation. The first simulation is a theoretical approach, whereas the second simulation processes experimental data. The first simulation allows the theoretical calculation of the isotope pattern of any single molecular or fragment ion given by the user. In addition, the isotopic pattern of a mixture composed of differently ^{13}C labelled variants of this species can be calculated. Hence, the program applies a recursive algorithm to calculate the isotope pattern of each molecular variant in a mixture. According to the abundance of each variant, the spectra are summed and then normalised. Due to the recursive nature of the algorithm, the program cannot calculate isotope patterns of molecules with more than approximately 50 C-atoms. However, for our calculations, which were for C_5–C_{10} compounds this was not a limiting factor. Other algorithms have been discussed in literature (Datta, 1997; Rockwood et al., 1995; Yergey, 1983). The relative abundances of molecular species were calculated using data from Chemstation software.

The second simulation implemented in the program allows calculation of the degree of labelling of a molecule variant mixture by entering the experimentally determined mass spectral data. Using measured data, the theoretical distribution of label across the molecule was calculated. When the measured and theoretical patterns were coherent, these data were used. The implemented algorithm was described by Biemann (1962). Results from the second simulation can be verified with the first simulation. The program is provided as freeware and can be downloaded at www.biology.kingdoms.ch/index.php?id=software.

4.8. Statistical analysis

Statistical analyses were performed using SPSS 16.0 for Mac OS X statistical package. The non-parametric Mann-Whitney U-tests were used to test differences between the percentage relative abundances of maximally labelled molecular species and non-labelled molecular species. Percentage data were transformed using arcsine transformation before statistical analysis. Differences were significant at $P < 0.05$ level.

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