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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.