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Isolation and structure elucidation of linolipins C and D, complex oxylipins from flax leaves

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ABSTRACT

Two complex oxylipins (linolipins C and D) were isolated from the leaves of flax plants inoculated with phytopathogenic bacteria *Pectobacterium atrosepticum*. Their structures were elucidated based on UV, MS and NMR spectroscopic data. Both oxylipins were identified as digalactosyldiacylglycerol (DGDG) molecular species. Linolipin C contains one residue of divinyl ether (ω5*Z*)-etherolenic acid and one α-linolenate residue at *sn*-1 and *sn*-2 positions, respectively. Linolipin D possesses two (ω5*Z*)-etherolenic acid residues at both *sn*-1 and *sn*-2 positions. The rapid formation (2–30 min) of linolipins C and D alongside with linolipins A and B occurred in the flax leaves upon their damage by freezing–thawing.

1. Introduction

Jasmonates and related oxygenated fatty acids, collectively called oxylipins, function in plants as plant hormones, signal molecules and potent antipathogenic compounds (Kazan and Manners, 2008; Mosblech et al., 2010). Their biosynthesis from polyenoic fatty acids is initiated by lipoxygenases (LOXs) followed by the transformations of hydroperoxy products by several CYP74 family (P450 superfamily) enzymes. This family includes the allene oxide synthase (CYP74A, CYP74C), hydroperoxide lyase (CYP74B, CYP74E, CYP74F and CYP74C) and divinyl ether synthase (DES) (Grechkin, 2002; Hughes et al., 2009).

DESs (CYP74D, CYP74H and CYP74B) control the dehydration of fatty acid hydroperoxide to divinyl ether fatty acids, featuring the ether oxygen incorporated into the carbon chain (Grechkin, 2002; Hughes et al., 2009). DESs are less common than other CYP74 enzymes. At the same time, DESs and divinyl ethers were detected in a big variety of plant species representing the distant taxons (Grechkin, 2002). For instance, DESs are present in some monocotyledonous species like garlic (Grechkin et al., 1995, 1997; Stumpe et al., 2008) and Lily-of-the-Valley (Ogorodnikova et al., 2008), as well as some dicotyledons, including members of Solanaceae (Grechkin, 2002; Hughes et al., 2009), Ranunculaceae (Hamberg, 1998, 2002, 2004) and Linaceae (flax) (Chechetkin et al., 2008; Gogolev et al., 2012). The divinyl ethers have also been detected in the brown alga *Laminaria sinclairii* (Proteau and Gerwick, 1993) and the red alga *Polyneura latissima* (Jiang and Gerwick, 1997). Divinyl ethers possess antimicrobial properties and play a role in plant defence toward microbial and viral infections, see (Gogolev et al., 2012) for references. As shown recently, DES gene silencing leads to pathogen susceptibility in *Nicotiana benthamiana* plants (Balaji et al., 2011).

The majority of known oxylipins are free oxygenated fatty acids or their macrolactones, esters or products of chain cleavage like aldehydes and aldoacids. Occasionally oxylipins also occur in plants as the constituents of complex lipids, for instance galactolipids. Presumably the first galactolipids-esterified oxylipins were detected in the red alga *Gracilariopsis lemaneiformis* (Jiang and Gerwick, 1990, 1991). These were three digalactosyl diacylglycerol

Abbreviations: DES, divinyl ether synthase; ESI MS, electrospray ionization mass spectrometry; (13*S*)-HPOT, (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid; (13*S*)-hydroperoxy-MGDG, 1,2-Di-*O*-[(9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoyl]-3-*O*-β-D-galactopyranosyl-*sn*-glycerol; LOX, lipoxygenase.

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(DGDG) molecular species possessing the esterified residues of vic-diol (2*R*,5*Z*,8*Z*,10*E*,l3*S*,14*Z*,17*Z*)-dihydroxyeicosa-5,8,10,l4,17-pentaenoic acid or the aldehyde (6*E*,8*E*,10*E*)-5-hydroxy-12-oxo-dodeca-6,8,10-trienoic acid (Jiang and Gerwick, 1990, 1991). Several galactolipid estolides containing the residues of avenoleic acid were detected in oat seeds (Hamberg et al., 1998; Moreau et al., 2008). The arabidopsides, galactolipids containing the residues of 12-oxo-10,15-phytodienoic acid and/or its 2,3-dinor homologue were detected in the leaves of *Arabidopsis thaliana* (Andersson et al., 2006; Hisamatsu et al., 2003, 2005; Kourtchenko et al., 2007; Nakajyo et al., 2006; Stelmach et al., 2001) and *Ipomoea tricolor* (Ohashi et al., 2005). Recently we have detected a novel family of complex oxylipins named linolipins in the flax (*Linum usitatissimum*) leaves (Chechetkin et al., 2009). First members of this family were identified as the monogalactosyl diacylglycerol (MGDG) species possessing the esterified residues of divinyl ether (ω5*Z*)-etherolenic acid. Linolipin B has two (ω5*Z*)-etherolenic acid residues both at *sn*-1 and *sn*-2 positions. Linolipin A possesses the α-linolenate residue and the (ω5*Z*)-etherolenic acid residue at *sn*-1 and *sn*-2 positions, respectively (Chechetkin et al., 2009). Here we report the isolation of two new members of linolipin family, linolipins C and D, and their identification as DGDG molecular species.

2. Results

2.1. Detection of linolipins in the infected and damaged flax leaves

Galactolipids were isolated from the flax leaves at 12 h after the inoculation of plants with cells of phytopathogenic bacterium *Pectobacterium atrosepticum* and analyzed by RP-HPLC. The analysis revealed four galactolipid molecular species exhibiting λ_{max} at 267 nm, thus indicating the possible presence of the esterified divinyl ether moieties (Fig. 1b). The retention times of compounds **1** and **3**, as well as their UV absorption, mass spectral and NMR data were identical to those of authentic standards of linolipins A and B (Chechetkin et al., 2009). Compound **4** had the same retention time as the previously detected pathogen-inducible galactolipid from flax leaves (compound numbered **3** in (Chechetkin et al., 2009)). Compound **2** has not been described yet.

The galactolipid molecular species **1**–**4** were also detected in the freeze–thaw injured flax leaves (Fig. 1c–e). The ratio of these compounds dramatically changed during 30 min after thawing of the frozen flax leaves. While compounds **1** and **2** were the predominant linolipins in the leaves at 1 min after thawing (Fig. 1c), their content at 30 min after thawing appeared to be about twice lower than the levels of compounds **3** and **4** (Fig. 1e). The proportions of compounds **1**–**4** stabilized at 30 min after thawing and then remained unchanged during the next 1 h (data are not illustrated).

Compounds **2** and **4** were collected and finally purified by cyanopropyl phase HPLC for further structural elucidation.

2.2. Identification of compound **2**, linolipin C

Pure compound **2** possessed a UV absorption spectrum identical to those of the divinyl ether (ω5*Z*)-etherolenic acid as well as the linolipins A and B, with the absorption maximum at 267 nm in MeOH methanol (Chechetkin et al., 2008, 2009). Transesterification of compound **2** with sodium methoxide afforded the methyl esters of α-linolenic acid and (ω5*Z*)-etherolenic acid as judged by the data of GC–MS analyses (not shown).

The electrospray ionization mass spectrum of compound **2**, recorded in the negative ion mode (Fig. 2b and Table S1), exhibited a quasimolecular ion $[M-H]^-$ at *m/z* 949.5472 ($C_{51}H_{81}O_{16}$), as well as the adduct $[M+CH_3COO]^-$ at *m/z* 1009.5791 ($C_{53}H_{85}O_{18}$). The obtained high resolution electrospray ionization mass spectral (ESI MS) data revealed the empirical formula $C_{51}H_{82}O_{16}$ for compound **2**. The MS/MS spectrum of $[M-H]^-$ ion showed several diagnostic ions (Fig. 2 and Table S1). The daughter ions at *m/z* 689.3406, 675.3407, 671.3298 and 657.3474 provide information about the fatty acid composition. The first pair of ions is consistent with the neutral losses of dehydrated α-linolenic and (ω5*Z*)-etherolenic acid residues (respectively) via the cleavage of ester bridges. The second pair results from neutral losses of the corresponding fatty acid residues. The relative abundance of ions at *m/z* 689.3406 and 671.3298 compared with ions at *m/z* 675.3407 and 657.3474 (Fig. 2b) indicates that (ω5*Z*)-etherolenic acid is esterified at *sn*-2 position, since the *sn*-2 substituents are eliminated more easily (Murphy and Harrison, 1994). The neutral losses of both *sn*-1 and *sn*-2 substituents resulted in the formation of negative ions at *m/z* 379.1231, 397.1356 and 415.1454 (Fig. 2 and Table S1), suggesting the presence of digalactosylglycerol moiety in compound **2**.

The positive ion mode mass spectrum of compound **1** (Table S1) exhibited the adduct $[M+NH_4]^+$ at *m/z* 968.5985 ($C_{51}H_{86}O_{16}N$). MS/

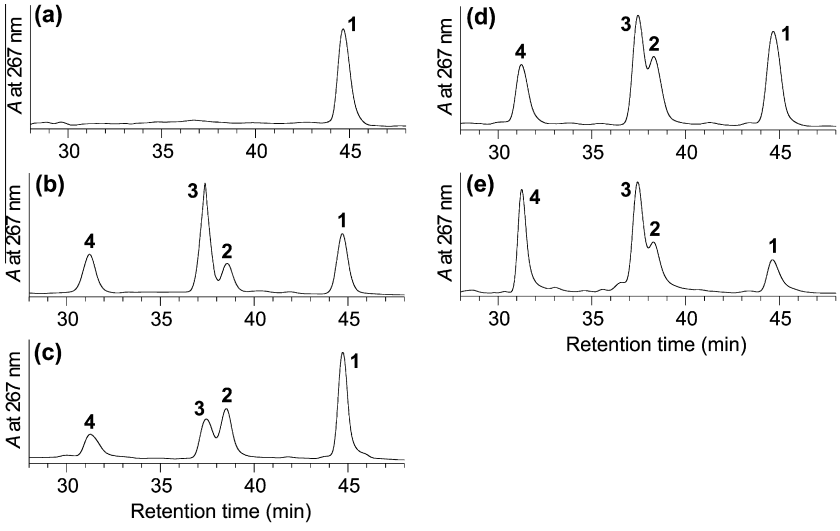

Fig. 1. The RP-HPLC profiles of galactolipid molecular species from flax leaves. Total galactolipids were extracted from flax leaves, separated and purified as described in the Materials and Methods. UV chromatograms (267 nm) of galactolipids extracted from: (a), control plants; (b), infected plants (at 12 h after inoculation with *P. atrosepticum*); (c), injured leaves (at 1 min after freezing–thawing); (d), injured leaves (at 2 min after freezing–thawing); (e), injured leaves (at 30 min after freezing–thawing).

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