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# Formation of oxidized phosphatidylinositol and 12-oxo-phytodienoic acid containing acylated phosphatidylglycerol during the hypersensitive response in Arabidopsis

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ABSTRACT

Plant membranes are composed of a wide array of polar lipids. The functionality of these extends far beyond a pure structural role. Membrane lipids function as enzyme co-factors, establish organelle identity and as substrates for enzymes such as lipases and lipoxygenases. Enzymatic degradation or oxidation (enzymatic or non-enzymatic) of membrane lipids leads to the formation of a diverse group of bioactive compounds. Plant defense reactions provoked by pathogenic microorganisms are often associated with substantial modifications of the lipidome. In this study, we profiled changes in phospholipids during the hypersensitive response triggered by recognition of the bacterial effector protein AvrRpm1 in *Arabidopsis thaliana*. A simple and robust LC–MS based method for profiling plant lipids was designed to separate all the major species of glycerolipids extracted from Arabidopsis leaf tissue. The method efficiently separated several isobaric and near isobaric lipid species, which otherwise are difficult to quantify in direct infusion based profiling. In addition to the previously reported OPDA-containing galactolipids found to be induced during hypersensitive response in Arabidopsis, three OPDA-containing sulfoquinovosyl diacylglycerol species, one phosphatidylinositol species as well as two acylated OPDA-containing phosphatidylglycerol species were found to accumulate during the hypersensitive response in Arabidopsis. Our study confirms and extends on the notion that the hypersensitive response in Arabidopsis triggers a unique profile of Allene Oxide Synthase dependent oxidation of membrane lipids. Primary targets of this oxidation seem to be uncharged and anionic lipid species.



## Introduction

Plant cellular and organellar membranes are composed of a large and diverse array of polar lipids. Apart from their obvious structural role, they also serve as precursors for signaling compounds and regulators of many different cellular processes (Wang, 2004). Phospholipase C (PLC) and D (PLD) which cleaves off the headgroup of glycerophospholipids leaving diacylglycerol (DAG) or phosphatidic acid (PA), respectively, are involved in intracellular signaling. In plants, PLC and PLD activity are linked to various biotic and abiotic stress responses (Li et al., 2009; Testerink and Munnik, 2011; Wang, 2004). Plant membrane lipids are rich in polyunsaturated fatty acids susceptible to oxidation by enzymatic or non-enzymatic processes. The oxidation of polyunsaturated fatty acids gives rise to a plethora of oxidized products collectively known as oxylipins (Andreou et al., 2009), among these the best understood are the members of the jasmonate family of plant hormones (Browse, 2009; Gfeller et al., 2010; Schaller and Stintzi, 2009). The oxylipins constitute both free fatty acids or fatty acid fragments and glycerolipid bound acyl groups. Generally, the role of oxylipins esterified to complex lipids is much less understood than that of the free fatty acids and fatty acid fragments. Among the lipid bound oxylipins are the so called arabidopsides which are chloroplast galactolipids containing enzymatically formed 12-oxo-phytodienoic acid (OPDA) and/or the C16 analog dinor-OPDA (Andersson et al., 2006a; Buseman et al., 2006; Hisamatsu et al., 2003, 2005; Kourtchenko et al., 2007).

Plants recognize and defend themselves against phytopathogenic microorganisms by a complex layered system (Jones and Dangl, 2006). A first layer is based on the ability to recognize typical microbial molecular structures known as Microbe Associated Molecular Patterns (MAMPs) (Monaghan and Zipfel, 2012). Plant pathogens have adapted to overcome the defense mounted after

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**Table 1**
Fragmentation modes used for different lipid classes.

| Lipid class | Adduct | Transition type | Transition (*m*/*z*) |
|---|---|---|---|
| MGDG ("normal" fatty acids and OPDA containing) | $NH_4^+$ | Neutral loss | 179[a] |
| DGDG ("normal" fatty acids and OPDA containing) | $NH_4^+$ | Neutral loss | 341[a] |
| SQDG | –H | Precursor | 225[b] |
| PC and lyso-PC | $H^+$ | Precursor | 184[c] |
| PE | $H^+$ | Neutral loss | 141[c] |
| PG | $NH_4^+$ | Neutral loss | 189[d] |
| PA | $NH_4^+$ | Neutral loss | 115[e] |
| PI | $H^+$ | Neutral loss | 260[f] |
| Acylated MGDG species | $NH_4^+$ | Neutral loss | 179 + acyl group[g] |

Head group specific fragmentations of phospho- and galactolipids reported in the literature, used in this study for construction of species specific MRMs as listed in Table S1.
[a] Isaac et al. (2007).
[b] Gage et al. (1992) and Welti et al. (2003).
[c] Brügger et al. (1997).
[d] Taguchi et al. (2005).
[e] Li-Beisson et al. (2010).
[f] Cole and Enke (1991).
[g] Ibrahim et al. (20110.

MAMP recognition. To this end the pathogens use secreted proteins known as effectors (Bent and Mackey, 2007). However, plants have in turn developed systems for recognition of the pathogenic effectors by so called R proteins. Recognition of pathogenic effectors often leads to a strong resistance response that frequently culminates in the so called hypersensitive response (HR) and programmed cell death. Previous studies indicate the involvement of phospholipases C (PLC) and D (PLD) in both MAMP and effector triggered responses (Andersson et al., 2006b; Kirik and Mudgett, 2009; Pinosa et al., 2013; van der Luit et al., 2000; Wang, 2004). Production of various oxylipins has also been reported in conjunction with primarily the HR (Andersson et al., 2006a; Kourtchenko et al., 2007; Montillet et al., 2005; Rusterucci et al., 1999; Vu et al., 2012; Zoeller et al., 2012). We have previously reported that PLD and PLC are involved in signaling triggered after recognition of the *Pseudomonas syringae* effector AvrRpm1 by the R-protein RPM1 and the effector R-protein pair AvrRpt2 and RPS2 (Andersson et al., 2006b). In addition, (dn)OPDA-containing galactolipids have been shown to accumulate in Arabidopsis tissue undergoing the HR (Andersson et al., 2006a; Kourtchenko et al., 2007; Vu et al., 2012; Zoeller et al., 2012). These are probably formed by the direct Lipoxygenase 2 (LOX2) and Allene Oxide Synthase (AOS) dependent oxidation and cyclisation of 16:3 and 18:3 bound to galactolipids (Glauser et al., 2009; Nilsson et al., 2012). Several other types of lipid bound oxidized and fragmented fatty acids were shown to accumulate in Arabidopsis tissue following the HR induced by *P. syringae* expressing AvrRpm1 (Zoeller et al., 2012).

There are several approaches available to profile complex lipids from plant tissue. Classically, complex lipids are separated into lipid classes by straight phase chromatography (HPLC or TLC) before hydrolysis and analysis of the liberated fatty acids by GC or HPLC. This quickly becomes cumbersome and it is usually not possible to entirely reconstruct the species composition of the lipid classes. Modern approaches to lipid profiling utilize tandem mass spectrometry, where specific scan modes are used to detect different lipid classes using head group specific fragmentation. A total lipid extract can be directly injected into the electrospray ion (ESI) source of a triple quadrupole mass spectrometer and using head group specific scans to construct a total lipid profile (Brügger et al., 1997; Isaac et al., 2007; Taguchi et al., 2005; Welti et al., 2003). This approach is highly efficient and has facilitated large advances in plant lipid research. However, direct infusion into the mass spectrometer comes with certain limitations. A complex mixture causes ion suppression in the source which has to be accounted for by the use of multiple internal standards. The common plant lipid molecular species are separated by only one or two unsaturations, which require isotopic deconvolution. Moreover, many lipid species are nearly or truly isobaric and thus impossible or difficult to separate in direct infusion experiments. Coupling mass spectrometry to online separation by HPLC alleviates many of these problems and several systems for both reverse and straight phase separation of membrane lipids have been described (see for example (Ibrahim et al., 2011; Laaksonen et al., 2006; Okazaki et al., 2013; Retra et al., 2008)).

In this study we developed a simple and robust method using ESI-MS coupled to reverse phase HPLC for profiling plant glycerolipids. The method was used to investigate AOS dependent changes in cellular phospholipids during the HR induced by the bacterial effector AvrRpm1. This lead to the identification of unexpected oxidized derivatives of phosphatidylglycerol (PG) and phosphatidylinositol (PI).

## Results

### *Development of a LC–MS method for profiling plant glycerolipids*

We intended to extend our previously used galactolipid separation method (Kourtchenko et al., 2007; Nilsson et al., 2012) to a wider range of glycerolipids and use tandem mass spectrometry to profile membrane lipids during the HR in Arabidopsis tissue. To this end we used head group specific multiple reaction monitoring (MRM) for the major Arabidopsis glycerolipids (Isaac et al., 2007) and the acylated MGDG species (Ibrahim et al., 2011) (Table 1). A gradient system previously used for galactolipids (Nilsson et al., 2012) initially seemed promising for separation of phospholipids as well. However, in our hands C18-columns never provided satisfactory separation of phosphatidylcholine (PC) species using the solvent system composed of acetonitrile:water and 2-propanol, whereas a C8 column gave sharp symmetric peaks of PC species. Change to a C8 column shortened the retention and thus an initial solvent mixture with a higher water content of the initial mobile phase was required to obtain good separation of OPDA-containing MGDG species. A gradient from Solvent A (acetonitrile:methanol:water, 35:35:30 by vol.) to 2-propanol was used. To facilitate ionization, both solvents were supplemented with formic acid and ammonia. We next aimed to obtain good separation of phosphatidic acid (PA) species. Initially, chromatographic performance of this lipid was poor. An excess of formic acid and addition of a low concentration (5 μM) of phosphoric acid to the solvent as described (Ogiso et al., 2008) gave fully satisfactory separation of PA. Taken together, this provided good separation of all major known species of PC (Fig. 1A), phosphatidylethanolamine