Changes of lipid metabolism of *Arabidopsis thaliana* in response to oligochitosan treatment

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Abstract

Lipids are vital cellular constituents in plant, and lipid peroxidation metabolites are critical defence substances in plants. In this study, mass spectrometry along with projections to latent structures discriminant analysis (PLS-DA) was used to detect lipid metabolism changes in *Arabidopsis thaliana* in response to oligochitosan (an effective resistance elicitor for the control of plant diseases). The PLS-DA showed that lipid metabolites of *Arabidopsis thaliana* were influenced by oligochitosan treatment. The total content of oxylipin containing monogalactosyldiacylglycerols, oxylipin-containing digalactosyldiacylglycerols, and oxylipin-containing phosphatidylglycerols increased firstly (after 1 h), and then decreased with the increase of oligochitosan treatment duration. In contrast, the total content of monogalactosyldiacylglycerols, phosphatidylcholines, phosphatidyl-ethanolamine, and phosphatidylglycerols decreased firstly, and then increased with the increase of oligochitosan treatment duration. The amounts of free fatty acids (C16:2, C16:3, C18:2, and C18:3) were lower after treatment with oligochitosan for 1, 3, and 6 h than in the control, while the production of volatile organic compounds such as 2-hexenal (except for 3 h) and nonanal was higher than in the control. In conclusion, lipid metabolites of *Arabidopsis thaliana* were influenced by oligochitosan treatment, and the synthesized lipids and oxylipin-containing lipids were remodelled and free fatty acids was metabolized to volatile organic compounds.

Additional key words: free fatty acids, oxylipin-containing lipids, volatile organic compounds.

Introduction

Lipids are vital cellular constituents that play important roles in transcriptional and translational control, cellular signalling, and cell-cell interactions (Wang 2004, Roberts et al. 2008). Galactolipids play an essential role in chloroplast development and the maintenance of electron transport systems, fatty acid synthesis, and photoreduction of cytochrome (Wang and Lin 2006). Phospholipids are major components of plasma membrane and organelle membranes that maintain the integrity of cells or organelles by creating a semi-impermeable barrier. Thus, lipid changes have impact on both cell structure and regulatory pathways during plant adaptations and survival. Lipid metabolism changes in plants are induced by various factors such as salinity, water stress, and extreme temperatures (Welti et al. 2002). Elkahoui et al. (2004) reported that phosphatidylcholine and phosphatidylethanolamine content increases and fatty acid composition is clearly modified when *Catharanthus roseus* is treated with NaCl. *Arabidopsis* maintains its polar lipid content and composition under severe water stress (Gigon et al. 2004). Wang et al. (2006) reported that the content of phospholipids of *Arabidopsis* increases to enhance membrane fluidity and to reduce propensity of cellular membranes in response to low temperatures. Therefore, lipid metabolism plays an important role in response to changes in the external environment.

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Abbreviations: BHT - butylated hydroxytoluene; DGDG - digalactosyldiacylglycerol; FFAs - free fatty acids; lyso-DGDG - lyso-digalactosyldiacylglycerol; lyso-MGDG - lyso-monomagalactosyldiacylglycerol; lyso-PG - lyso-phosphatidylglycerol; MGDG - monogalactosyldiacylglycerol; PC - phosphatidylcholine; PE - phosphatidylethanolamine; PG - phosphatidylglycerol; PI - phosphatidylinositol; PLS-DA - projections to latent structures discriminant analysis; UPLC - ultra performance liquid chromatography.

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Elicitors have no antimicrobial activity themselves, but they induce resistance or prime a plant for future pathogen attack (Liaqata and Eltem 2018). Elicitor also can induce lipid metabolic changes in plants. Kupper et al. (2006) reported that polysaccharides could induce the release of free saturated and unsaturated fatty acids and the accumulation of oxylipins in Laminaria digitata. Agarooligosaccharides induce changes of lipids metabolism in Pyropia haitanensis (Wang et al. 2014). As a type of biological elicitor, oligochitosan has been shown to be an effective resistance elicitor for the control of plant diseases (Yin et al. 2010). Oligosaccharides can induce gene expression and production of nitric oxide and H₂O₂ (Yin et al. 2006, Zhao et al. 2007a,b). Chen et al. (2009) reported that oligochitosan induces expression of gene for mosaic virus protein involved in resistance and pathogenesis in tobacco. However, oligochitosan induced changes of lipid metabolism are still not well understood in plants. Arabidopsis thaliana, a small cruciferous plant, is an ideal model for investigating various aspects of higher plant biology including plant and pathogen interactions (Meyerowitz et al. 1991, Meinke et al. 1998, Mauch-Mani and Slusarenko 1993). In the present study, we analyzed the changes in lipids of A. thaliana treated with oligochitosan. The present study aimed at understanding the qualitative and quantitative changes of lipids in A. thaliana in response to oligochitosan treatment. The knowledge of glycerolipid changes of A. thaliana in response to an elicitor will facilitate the understanding of membrane lipids in plants.

Materials and methods

Chemicals and reagents: Acetonitrile, isopropanol, formic acid, and sodium formate for liquid chromatography-mass spectrometry (LC-MS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Distilled water was further treated with a Milli-Q system (Millipore, Bedford, MA, USA). Leucine-enekephalin was purchased from Sigma-Aldrich. Standards of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) were obtained from the Avanti Polar Lipids (Alabaster, AL, USA). Glycolipid standards including monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyl-diacylglycerol (SQDG) were purchased from Lipid Products (Redhill, Surrey, UK). Oligochitosan (average Mr of 5 000) was purchased from Sigma-Aldrich.

Plants, growth conditions, and treatments: Arabidopsis thaliana L. (genotype Col-1) seeds were kindly provided as a gift by Professor Zhongnan Yang from Shanghai Normal University. Seeds of A. thaliana were germinated in growth chambers set at a temperature of 23 ± 1 °C, a 16-h photoperiod, an irradiance of 144 μmol m⁻² s⁻¹, and a 70 % relative humidity. The soil was Vermiculite: peat: Perlite = 6:1:0.5. Thirty-day-old seedlings were used for further experiment. Oligochitosan induction experiment was performed on 30-d-old A. thaliana plant. Oligochitosan solution at concentration 50 μg cm⁻² was sprayed on the leaf of Arabidopsis thaliana to elicit defence responses. Samples were harvested at 0.5, 1, 3, and 6 h after treatment. The control group was sprayed with sterile water. Six replicates were performed and the leaf samples were stored at -80 °C for analysis.

Lipid analysis: Briefly, 50 mg of samples (freeze-dried) was extracted using chloroform/methanol/water (1:2:0.8, v/v/v) solution containing 0.05 % (v/v) butylated hydroxytoluene by a method reported by Bligh and Dyer (1959) with minor modifications. The samples were dried under nitrogen gas, and dissolved in methanol for ultra performance liquid chromatography-quadrupole-time of flight mass spectrometry (UPLC-qTOF-MS) analysis. Chromatographic separation was performed on an ACQUITY UPLC BEH C8 analytical column (100 × 2.1 mm, 1.7 μm, Waters, Milford, MA) using an ACQUITY UPLC system (Waters). Optimal separation was achieved with a gradient elution using A - water containing 0.1 % (v/v) formic acid and 0.01 % (v/v) lithium acetate; and B - a mixture of methanol: acetonitrile: iso-propanol (1:2:1, v/v/v, containing 0.1 % v/v formic acid and 0.01%, v/v, lithium acetate) at a flow rate of 0.45 cm² min⁻¹. The mobile phase B was held for 1 min at 5 %, then changed to 60 % in 1 min, reached 98 % in 18 min and held for 5 min, returned to the initial 5 % in 1 min and equilibrated for 4 min. The injection volume was 5 mm³. An aliquot of each sample was injected into the column and 25 % of the effluent was split into the mass spectrometer. Mass spectrometry analysis was according to Wang et al. (2014) and Li et al. (2014).

Analysis of free fatty acids by gas chromatography-mass spectrometry: Free fatty acids were extracted from the A. thaliana tissues according to Kupper et al. (2006) and dried under nitrogen gas. Fatty acids were methylated with boron trifluoride-methanol solution (14 % in methanol) for 1 h at 60 °C. The analysis of fatty acid esters was performed on an Agilent Technologies 7890A gas chromatography system fitted with a SPB-50 fused capillary silica column (30 m × 0.25 mm, 0.25 μm; Supelco, Bellefonte, PA, USA) coupled with an Agilent Technologies 5975C mass spectrometer. The injection temperature was 250 °C. After injection, the column temperature was kept at 100 °C for 3 min, before being increased to 230 °C at a rate of 10 °C min⁻¹ and kept for 10 min, and subsequently increased to a final temperature of 240 °C at a rate of 1 °C min⁻¹ and kept for 10 min. It was further increased to a final temperature of 280 °C at a rate of 5 °C min⁻¹ and kept for 15 min. The injection volume was 1 mm³ with a split ratio of 5:1. Mass spectrometry was operated under the electron impact mode with 70 eV of electron energy. The ion source temperature and interface temperature were set at 230 and 220 °C, respectively. The scan range was from m/z 45 to 450. The individual substances were identified on the basis of their retention times by comparing their mass spectra with those recorded in Nist 11 and DEMO. L Spectrometry Library and those related to the previous analysis of pure
Analysis of volatile organic compounds by gas chromatography-mass spectrometry: The solid phase microextraction of *A. thaliana* tissues was conducted according to Croisier et al. (2010). A solid phase micro extraction (SPME) fiber (Supelco, Bellefonte, PA, USA) coated with an absorbent phase made of polydimethylsiloxane/carboxen/divinylbenzene (PDMS/CAR/DVB) was exposed in the headspace at 50 ± 1 °C for 40 min. After sampling, the fiber was analyzed using gas chromatography-mass spectrometry on an Agilent Technologies 7890A gas chromatography system fitted with a Vocol column (60 m × 0.32 mm, film thickness 1.8 μm; (Supelco) coupled with an Agilent Technologies 5975C mass spectrometer. The oven temperature was programmed as follows: 35 °C for 3 min, then to 40 °C at 3 °C min⁻¹ and held for 10 min, finally to 210 °C at 3 °C min⁻¹ and held for 20 min. The injection was splitless and the injector temperature was 210 °C. The mass spectra were obtained under electron ionization impact at 70 eV and data acquisition was performed over an m/z range of 50 - 600. The analytes were identified on the basis of their retention times by comparing their mass spectra with those recorded in *Nist 11* and *DEMO. L Spectrometry Library* and those related to the previous analysis of pure references that are commercially available.

Data analysis: All the data were shown as means ± standard deviations (SDs). The preprocessing of UPLC-MS data was performed with MarkerLynx 4.1 software (Waters, Milford, MA, USA). Pareto-scaled MarkerLynx matrices, including the peak number (based on the retention time and m/z), sample name, and the normalized peak intensity, were analyzed by projections to latent structures discriminant analysis (PLS-DA) using the SIMCA-P+ software package (v. 12.0, Umetrics AB, Umetric, Umea, Sweden). For each sampling time, differences between the treatments and control were analyzed via one-way analysis of variance (ANOVA) and the Duncan multiple comparison test. Different lowercase letters were considered statistically significant at level *P* < 0.05. Analysis was undertaken using SPSS 13.0 (SPSS, Chicago, IL, USA) for Windows.

Results

Fourteen MGDGs (mainly C₁₈:₃/C₁₆:₃, C₁₈:₄-O/C₁₆:₄-O, and C₁₈:₃/C₁₈:₃), 3 lyso-MGDGs, 5 DGDGs (mainly C₁₈:₃/C₁₈:₃, C₁₈:₃/C₁₆:₃, and C₁₈:₃/C₁₆:₄), 1 lyso-DGDG, 5 SQDGs (mainly C₁₆:₀/C₁₈:₂), 11 PGs (mainly C₁₆:₀/C₁₈:₁ and C₁₆:₀/C₁₈:₂), 1 lyso-PG, 5 PCs (mainly C₁₈:₃/C₁₈:₂), 2 lyso-PEs, and 2 PIs were identified in the control Arabidopsis plants and plants treated with oligochitosan (Table 1 Suppl.). There was no difference in composition of MGDGs, lyso-MGDGs, DGDGs, lyso-DGDG, SQDGs, PGs, lyso-PG, PCs, PEs, lyso-PEs and PIs among control group and treatment groups after 1, 3, and 6 h (*P* > 0.05).

Using positive and negative ion scan modes, lipid metabolic changes of control and treatment groups were analyzed with PLS-DA (Fig. 1 Suppl.). The samples were classified into three clusters: lipid metabolic changes in *A. thaliana* treated with oligochitosan for 0.5 h, 3 h, and the control group were classified into one cluster, while lipid metabolic changes in *A. thaliana* treated with oligochitosan for 1 h or 6 h were classified into two separate clusters, respectively. The PLS-DA loading plot showed that oligochitosan treatment had an effect on the lipid profile of Arabidopsis thaliana.
ESI-MS/MS was used to analyze the changes of lipids in A. thaliana treated with oligochitosan (Table 1 Suppl. and Fig. 1). The total content of MGDGs decreased with oligochitosan treatment for 1 h ($P < 0.05$), and then gradually increased after treatment for 3 h ($P < 0.05$), finally returned to the control level after oligochitosan treatment for 6 h ($P < 0.05$). Compared to the control, the total content of oxylipin-containing MGDGs, oxylipin-containing DGDGs, and oxylipin-containing PGs increased after treatment with oligochitosan for 1 h ($P < 0.05$), and then decreased and were lower after 3 and 6 h than in control group ($P < 0.05$). No difference was found in total DGDGs content in all groups.

The total amount of PEs and PGs in A. thaliana treated with oligochitosan for 1, 3 h (except for PGs), and 6 h were lower than those in control group ($P < 0.05$), while no differences were found among oligochitosan treated groups (1, 3, and 6 h; $P > 0.05$). The total PC content decreased with oligochitosan treatment for 1 h ($P < 0.05$), and then gradually increased after 3 and 6 h, and the PCs content after 6 h was higher than in control group ($P < 0.05$).

Nine types of fatty acids (C14:0, C16:1, C16:2, C18:0, C18:1, C18:2, and C18:3) were analyzed by GC-FID (Fig. 2). No difference was found in the content of free fatty acids in A. thaliana treated with oligochitosan for 1, 3, and 6 h. A - C14:0, C16:1, C16:2, C18:0, C18:1; B - C16:0, C16:3, C18:2, and C18:3. Means ± SDs, n = 6. Different lowercase letters indicate significant differences (the Duncan's test, $P < 0.05$).

The effects of oligochitosan treatment on the amounts of volatile organic compounds in A. thaliana were also analyzed by GC-FID (Fig. 3). A - 2-penten-1-ol, 1-penten-3-one, 4-penten-1-ol, hexanal, 2-hexenal, 2,4-heptadienal, and 3-methyl-4-penten-1-ol. B - 2,6-nonadienal, nonanal, 4-heptanal, 2,4-hexadienal, 2-nonanal, 3-pentanone, 3-hexen-1-ol, and 6-methyl-5-hepten-2-one. Means ± SDs, n = 6. Different lowercase letters indicate significant differences (the Duncan's test, $P < 0.05$).
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c16:3, c18:0, c18:1, c18:2, and c18:3) in control and treated groups were identified (Fig. 2A, B). The c18:3 was the most abundant species, followed by c16:3 and c18:2 in both control and treated groups. The content of free fatty acids c16:0, c16:2, c16:3, c18:1, and c18:3 was lower in groups treated with oligochitosan for 1, 3, and 6 h than in control (P < 0.05), while there were no differences among oligochitosan treated groups (1, 3, and 6 h) (P > 0.05). The content of fatty acid c18:1 in plants treated with oligochitosan for 3 h was lower than in control and other treatments, while there was no difference in c18:1 content between control and groups treated for 1 and 6 h (P > 0.05). The c14:0 content in A. thaliana treated with oligochitosan for 1 h was lower than in control (P < 0.05), while there was no difference among those treated for 3, 6 h, and control (P > 0.05). The c18:0 content in plants treated with oligochitosan for 1 and 3 h was lower than in control (P < 0.05), while there was no difference between those treated for 6 h and control (P > 0.05). The content of c16:1 fatty acid in A. thaliana treated with oligochitosan for 6 h was lower than in control (P < 0.05), while there was no difference in among 1 h, 3 h, and control (P > 0.05).

Eight aldehydes, 3 ketones, and 4 alcohols were identified in both the treatment and control groups (Fig. 3A, B). 2-hexenal was the dominant volatiles in A. thaliana in all groups (Fig. 3A). Compared to the control, a number of volatile organic compounds increased after treatment with oligochitosan (Fig. 3). The content of 4-penten-1-ol, 2-hexenal, 2,4-heptadienal, and nonanal in the groups treated for 1, 3 (except for 2-hexenal), and 6 h were higher than in control group (P > 0.05). The content of 3-hexen-1-ol and 6-methyl-5-hepten-2-one in the group treated for 3 and 6 h was higher (P > 0.05) than that in the group treated for 1 h and control group. The content of 2-nonenal, 2-penten-1-ol, and hexanal in the group treated for 6 h was higher than that in groups treated for 1 or 3 h in control group (P < 0.05).

Discussion

Lipid metabolism changes can be induced by elicitors and stress (Pohnert 2002). In the present study, PLS-DA loading plot showed that oligochitosan treatment had an effect on the lipid profile of Arabidopsis thaliana. PLS-DA scores scatter plot showed that the metabolite profiles of A. thaliana in the control, and treatments groups (with oligochitosan for 0.5, 1, 3, and 6 h) can be classified into three clusters, suggesting that the lipid metabolism of A. thaliana was changed by oligochitosan. Moreover, the control group was clustered together with the groups treated with oligochitosan for 0.5 and 3 h. However, the lipid profile among the control group, group treated with oligochitosan for 1 h and group treated with oligochitosan for 6 h were significantly different. The results indicated that the oligochitosan did not cause lipid changes after 0.5 h, but the lipids started to change after 1 h, and then returned to the levels of the control group after 3 h. Interestingly, the lipids started to change again after 6 h.

Following biotic and abiotic stress, lipids can release FFAs (including free saturated fatty acids and free unsaturated fatty acids), and then the FFAs can be oxidized into a wide range of metabolites. The lipids can also be directly oxidized into oxylipin-containing lipids (Genva et al. 2018). In this study, the total amount of oxylipin-containing glycolipids (MGDGs and DGDGs) and oxylipin-containing PGs increased at 1 h after treatment with oligochitosan; however, glycolipids (MGDGs), PGs, and FFAs (C14:0, C16:0, C16:2, C16:3, C18:0, C18:2 and C18:3) decreased at the same time. The results suggested that the synthesized glycerolipids were oxidized to oxylipin-containing glycerolipids, rather than they released FFAs in response to oligochitosan treatment.

Plants produced high amount of oxylipin-containing lipids under stress conditions (Genva et al. 2018). Buseman et al. (2006) found that the content of oxylipin-containing lipids (glycerolipids containing oxophytodienoic acid and dinor-oxophytodienoeic acid) was higher in Arabidopsis wounded leaves than in control ones. 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 (Chechetkin et al. 2013). Wang et al. (2017) demonstrated that the content of oxylipin-containing MGDGs, oxylipin-containing DGDGs, and oxylipin-containing PGs in A. thaliana increased with the increased time after infection with Sclerotinia sclerotiorum. Total amount of oxylipin-containing DGDGs and MGDGs of A. thaliana leaves increased 5- to 6-fold at 45 min after wounding (Vu et al. 2014), while oligochitosan changes were much lower in A. thaliana after treatment with oligochitosan for 1 h (P < 0.05) in this study. The results indicated that oligochitosan was a protective elicitor, not an injury signal. With the increased time of treatment with oligochitosan, the total content of oxylipin-containing glycolipids, the total content of oxylipin-containing glycolipids (MGDGs and DGDGs) and oxylipin-containing PGs decreased at 3 and 6 h, and it was lower than that in the control group. These results could be explained as a strategy of protection against oxidative damage through membrane restructuring with less oxylipin-containing glycolipids (Bejaoui et al. 2016).

Free mono- and polyunsaturated fatty acids are further converted to short chain volatile components (Feusner and Wasternack 2002, Stumpe et al. 2006). For example, C18:2 and C18:3 fatty acids are converted into hexanal, 2-hexenal or 3-hexenal, and 2-nonenal or 2,6-nonadienal (Matsui et al. 2006, Gigot et al. 2010). In this study, we found that the content of free fatty acids (C16:2, C16:3, C18:2, and C18:3) in A. thaliana after treatment with oligochitosan (1, 3, and 6 h) was lower than that in control, suggesting that the released FFAs might be further metabolized, resulting in the decline of their relative content. The emission of volatile organic compounds was observed in this study. For example, the content of 2-hexenal and nonanal in A. thaliana after treatment with oligochitosan for 1, 3 (except for 2-hexenal), and 6 h was higher than in the control group. Thus, the FFAs (C16:2, C16:3, C18:2, and C18:3) would be metabolized to volatile organic compounds in response to oligochitosan treatment. Most plant volatile organic compounds have bacteriostatic activity, which act as plant resistance and...
chemical defense factors against pathogen infection (Scalia et al. 2013). Vaughn and Gardner (1993) reported that 2-hexenal and 2-nonenal inhibit growth of Rhizoctonia solani and Sclerotium rolfsii. Kishimoto et al. (2006) reported that 2-hexenal enhances resistance of Arabidopsis thaliana against a necrotrophic pathogen Botrytis cinerea. Nonanal, 2-nonenal, and 2,6-nonadienal have fungicial activities against Botrytis cinerea and Fusarium oxysporum (Matsui et al. 2006). In this study, we also found that amount of these volatile organic compounds increased. For example, 2-nonenal and hexanal content in A. thaliana after treatment with olioigochitosan for 6 h was higher than that in control group, suggesting that some volatile compounds could participate in the induction of resistance in A. thaliana.

In summary, the lipid metabolism was activated in response to olioigochitosan treatment, as shown by the changes in glycerolipids and oxylipin-containing glycolipids, via decreasing the FFAs content and increasing content of volatile organic compounds.

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