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Original Communication

Cellular phospholipid degradation during viability loss in recalcitrant seeds of *Quercus glauca* (Fagaceae)

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ABSTRACT

This study is aimed to determine the relationship between drying, seed physiology, oxidative stress-induced lipid peroxidation and chemistry of lipid peroxidation products during drying and viability loss of *Quercus glauca* seeds. We used labeled phosphatidylcholines, LA-PC/D_3 **2** and DHA-PC/Et **3**, as molecular probes, and analyzed them using tandem electrospray ionization mass spectrometry (ESI MS). When the seeds of *Quercus glauca* (Fagaceae) were slowly dried to 50.6%, 36.7% and 23.1% water content, they germinated to 90%, 62% and 2%, respectively, indicating this species produces desiccation-sensitive seeds. During drying, the amounts of 2-thiobarbituric acid reactive substances (TBARS, a marker of lipid peroxidation), significantly increased in the seeds, and then decreased, in line with loss of viability. However, TBARS are terminal compounds formed from phospholipids via multiple steps, and the chemistry is complex. The experiments revealed that the extent of the oxidative decomposition of synthetic phospholipid **3** added to *Q. glauca* seed embryos was dependent on seed moisture content, the exposure time of the phospholipid and embryo mass. The significant correlations between the moisture content, embryo lipid peroxidation and seed viability loss indicate that desiccation stress resulted in viability loss due to cell membrane damage. These results are consistent with reactive oxygen species (ROS)-mediated lipid peroxidation determining the physiology of the drying-sensitive seeds.

KEYWORDS: recalcitrant seeds, phospholipid, molecular probe, ESI MS, reactive oxygen species, *Quercus glauca*

INTRODUCTION

Extensive studies have accumulated vast arrays of evidence that oxidative stress-induced lipid oxidation is causally involved in pathological events in biological systems including plants [1] and mammals. Recent studies have also revealed that some lipid peroxidation products exhibit unexpectedly broad biological activities including potential beneficial pathological properties [2, 3]. These newly emerged aspects of lipid peroxidation are of intrinsic interest justifying further investigations and discussions.

The extent of seed desiccation tolerance differs among species. Based on their response to desiccation, seeds can be divided into two broad groups: orthodox and recalcitrant [4]. Orthodox seeds can be dried to low water content (< 7%) with little impact on viability [5]. In contrast, recalcitrant seeds would lose viability when dried to water content as high as 20-30% [6]. Thus, recalcitrant seeds are desiccation-sensitive. One of the mechanisms or processes linked to desiccation-induced seed deterioration and death is the over-accumulation of reactive/active oxygen

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species (ROS/AOS). This can result in oxidative damage to lipids, proteins and nucleic acids during desiccation of seeds, in particular, dehydration-intolerant recalcitrant seeds [7, 8, 9]. The decline in seed viability with desiccation is accompanied by an increase in the thiobarbituric acid-reactive substances (TBARS) resulting from lipid peroxidation [10]. Measuring electrical conductivity of the steep water from desiccated seeds by means of some traditional methods has also demonstrated relations between seed activity and free radical level [10, 11]. There are inherent technical difficulties at the molecular level that have limited the study of oxidative stress-induced lipid peroxidation and the chemistry of lipid peroxidation products. Our technique combining tandem electrospray ionization mass spectrometry (ESI MS) with synthetic phospholipid hydroperoxides **2** and **3** as molecular probes for natural phospholipid hydroperoxide **1** [12], was confirmed rapid and accurate in this study. The molecules of phospholipid hydroperoxides **2** and **3** are the typical first molecular species formed during chemical or biological lipid peroxidation induced by inner mechanisms. An ethyl group in the structure of **3** replaces one of the *N*-methyl groups in the structure of **1**.

MATERIALS AND METHODS

Seed collection and viability test

Mature seeds of blue Japanese oak (*Quercus glauca* Thunb., Fagaceae) were collected at the Okayama University, Japan (34°39.6′ N, 133°55.6′ E, Altitude 28 m) on December 3, 2008. These seeds were spread in a single layer on the laboratory desktop to be slowly dried at about 15 ± 3 °C and ~ 50% relative humidity. Seed characteristics were measured on 50 randomly selected seeds. At intervals, samples of 10 seeds or 30 isolated embryos were used to measure gravimetric moisture content. The embryos were dried at 103 °C for 17 h in an oven before the measurement [13]. All moisture contents were recorded on a fresh weight basis. Four replicates of 25 seeds were placed on a sheet of moist filter paper in a Petri dish at 26 °C in light. Seeds were considered germinated when the radical emerged from the seed coat. Germination tests lasted 30 days.

Role of the synthetic phospholipids 2 and 3 in the present study

In collision-induced tandem ESI MS, since all the natural phosphatidylcholines (PC) such as **1** afford a product ion at *m/z* 184, which is a mass to charge ratio of natural choline phosphate moiety in **1** (Fig. 1), the precursor ion scan mode at *m/z* 184 afford signals of all the natural PC. On the other hand, when *m/z* 187 is used instead of 184, signals of **2** and its degradation products that have the same unnatural polar head group can be detected exclusively in a strictly selective manner even in the presence of excess of complex natural PC. Therefore, **2** can be used as a molecular probe to trace the fate of PC in complex biological systems such as seed embryo in the present study and human blood as in our previous study [12]. Also, since another synthetic phospholipid **3** results in a product ion at *m/z* 198, the precursor ion scan mode at *m/z* 198, instead of 184 or 187 can detect **3** and its degradation products

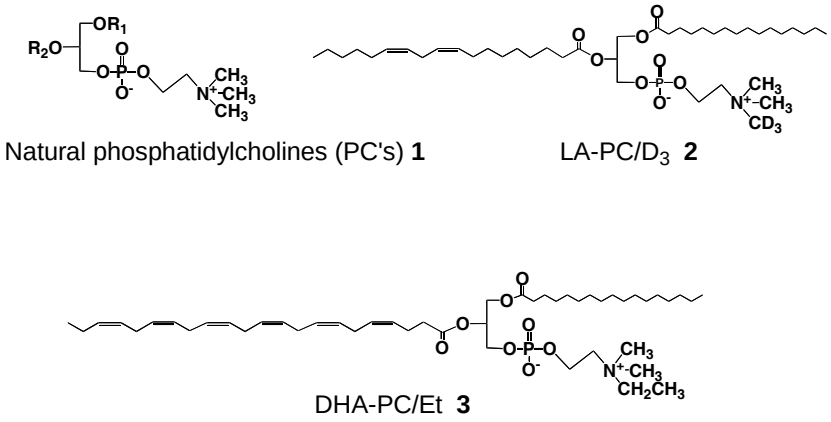

Fig. 1. Chemical structure of natural phosphatidylcholines (PC) **1**, molecular probes **2** and **3**.

exclusively. Therefore, these unique molecular probes can play key roles in the unambiguous detection of specific lipid peroxidation *in vivo*.

ESI MS condition

Mass spectrometric analyses were performed with a Perkin-Elmer SCIEX (Thornhill, ON, Canada) API-III tandem quadrupole mass spectrometer. A mixture of $CH_3CN/CH_3OH/H_2O$ (215:194:16) with 0.1% ammonium acetate was used as a solvent, and the sample solution was introduced by direct infusion for spectral acquisition. LA-PC/D_3 **2** was synthesized using deuteriomethylcholine tosylate instead of choline tosylate [12]. Briefly, 1-palmitoyl-*sn*-lysophosphatidylcholine was prepared via lipase-catalyzed acylation of 2-*O*-benzylglycerol, introduction of 2-(*N*, *N*-dimethyl-*N*-trideuteriomethyl)ethyl phosphate using phosphorus oxychloride and 2-hydroxy-(*N*, *N*-dimethyl-*N*-trideuteriomethyl) ammonium *p*-toluenesulfonate under standard conditions. This was followed by the removal of the benzyl group by Pd-catalyzed hydrogenolysis, and esterification with docosahexaenoic acid using dicyclohexylcarbodiimide and dimethylaminopyridine. DHA-PC/Et **3** was synthesized by the same method using 2-hydroxy-(*N*, *N*-dimethyl-*N*-ethyl) ammonium *p*-toluenesulfonate instead of 2-hydroxy-(*N*, *N* -dimethyl-*N*-trideuteriomethyl) ammonium *p*-toluenesulfonate.

Lipid peroxidation

Lipid peroxidation products of intact seeds at different moisture contents were determined by measuring the amounts of TBARS in freshly prepared extracts by the method of Heath and Packer [14], and were quantified using 1, 1, 3, 3-hydroxypropane as a standard. A trace of butylated hydroxytoluene (BHT, 2% w/v) was included in the reaction mixture to eliminate artifactual peroxidative damage to the samples during processing, as described by Hendry *et al*. [15]. Three replicate samples were assayed.

Reaction of LA-PC/D_3 2 with desiccated embryos

In this experiment, 25 mg of each isolated embryo sample with 36.7% water content (dried for 9 days) was placed in a 1 ml tube with 50 µl LA-PC/D_3 **2** (25 µM) dissolved in a mixture of ethanol and water (1:1, v/v), and were stirred at intervals for 20 h at room temperature. The reaction mixture was analyzed by precursor ion scan mode in ESI MS.

Effect of dried embryo on DHA-PC/Et 3

Samples of about 25 mg isolated embryos at 57.7, 50.1, 36.7, 30.2 and 23.1% water content were placed in 1 ml tubes containing 50 µl DHA-PC/Et **3** (23.9 µM) dissolved in a mixture of ethanol and water (1:1, v/v), and were stirred at intervals for 20 h at room temperature. A trace of BHT was then added to stop the reaction. Finally, 20 µl of the reaction mixture diluted with a mixture of $CH_3CN/CH_3OH/H_2O$ (215:194:16) (100 µl) and 0.1% ammonium acetate was submitted to measurement by tandem ESI MS.

Analysis of the degradation time-course of DHA-PC/Et 3

To study the effects of reaction time on DHA-PC/Et **3**, two groups of embryo having 50.6% water content were used in a series of experiments. One group was exposed to nitrogen (N_2), and the other was exposed to air. In each group, 1 ml tubes containing about 40 embryos (100 mg) were allocated 0.5 ml mixture consisting of DHA-PC/Et **3** dissolved in ethanol/water (1:1, v/v). A time course was generated by adding a trace of BHT to different tubes at intervals of 1, 2, 4, 6, 8 and 20 h, to stop the reaction. These reaction mixtures were submitted to ESI MS as described above.

Effects of the amounts of embryo on DHA-PC/Et 3

Six groups of samples with different amounts of isolated embryos with different water content were used to estimate the effects of sample size on DHA-PC/Et **3**. Reaction time for each sample was 20 h under the similar conditions described above. The profile and extent of degradation of DHA-PC/Et **3** was determined by ESI MS as described above.

RESULTS

Seed characteristics

The characteristics of mature seeds of *Q. gluaca* can be summarized as follows: fresh weight of a single seed varies from 1.0 g to 2.2 g (mean, 1.61 ± 0.29 g), the average width, 1.68 cm; the average length, 2.17 cm; initial seed moisture content (MC), 40.9 ± 1.1%; initial embryonic MC, 57.7 ± 1.9%; initial cotyledon MC, 40.6 ± 2.3%. The embryo was much smaller (≈ 2.5 mg) than the cotyledon and therefore, the initial seed MC was almost equal to that of the cotyledon MC.

Effects of desiccation on seed viability

Seed moisture content decreased progressively with drying time to 23% MC over 21 days (Table 1). As expected for recalcitrant seeds, germination (viability) decreased with increasing desiccation. Initial germination of embryos (*ca*. 58% MC) was 98%, but reduced to 62% with drying to 36.7 ± 0.6% MC. These results showed that *Q. glauca* seeds were sensitive to (slow) desiccation, consistent with its classification as recalcitrant.

Lipid peroxidation

In *Q. glauca* seeds, there was significant increase (from 42 to 123 nmol g^{-1} dwt) in TBARS while drying to below 20% MC. However, further drying to 13.9% MC reduced TBARS to 61 nmol g^{-1} dwt, which was a point where most seeds lost viability (Fig. 2).

Reaction of LA-PC/D_3 2 with dried embryos

LA-PC/D_3 was submitted to reaction with embryos that had been dried for 9 days. The lipid extract was analyzed by precursor ion scan mode in tandem ESI MS at *m/z* 187 as a product ion. The spectrum shown in Fig. 3, indicated a number of signals whose *m/z* was lower than that of undecomposed LA-PC/D_3 (expected *m/z* is 761.6 for $[C_{42}H_{77}D_3NO_8P+H]^+$). This indicates that the molecular species for the observed *m/z* had lower molecular weight than that of LA-PC/D_3. Since unconjugated *cis*, *cis* double bond structure in the linoleoyl group of LA-PC/D_3 (Fig. 1) is sensitive to oxidation with reactive oxygen species, this suggests that these species are molecular fragments formed from LA-PC/D_3 via oxidative degradation. Molecular structures (Fig. 4) were deduced from the observed *m/z* and well-documented mechanisms for the oxidation of polyunsaturated olefinic structure.

Table 1. Drying time, embryonic moisture content, germination percentage and relative intensity of a peak of remaining DHA-PC/Et **3** after 30 h reaction with embryos at room temperature. Values are means (± SD) of the three replicates of 20 embryos.

Drying time (days)	Embryonic moisture content (% of fresh weigh) (SD)	Germination (%)	Relative intensity (%) of the peak of remaining DHA-PC/Et **3**
0 (Control)	57.7 ± 1.9	98 ± 0.7	58.7 ± 2.9
2	50.6 ± 0.7	90 ± 2.8	34.8 ± 1.2
9	36.7 ± 0.6	62 ± 2.1	26.3 ± 1.1
14	30.2 ± 2.7	38 ± 2.1	13.8 ± 0.1
21	23.1 ± 2.2	2 ± 1.4	10.2 ± 0.1

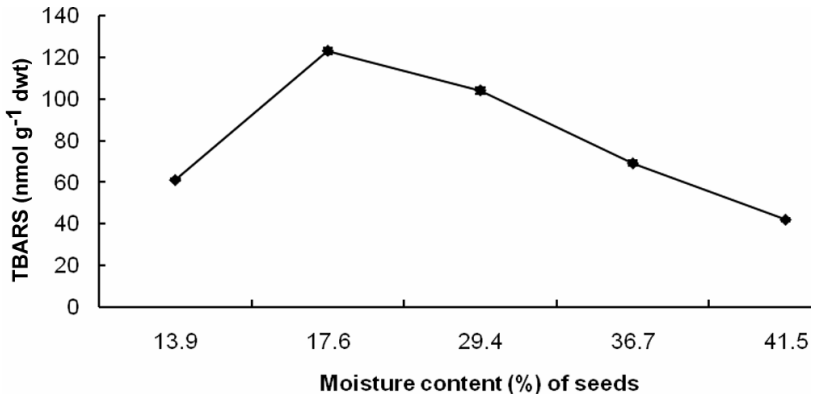

Fig. 2. Changes of lipid peroxidation measured as the amounts of thiobarbituric acid reactive substances (TBARS) during seed desiccation. Values are the mean of three replicates ± SD.

Changes in relative intensity of DHA-PC/Et 3

Embryos that underwent different drying times resulted in different relative intensities when mixed with DHA-PC/Et (Table 1). With fresh embryos (57.7% MC), the relative intensity of the remaining peak was 58.7%. When embryos with 23.1% MC were simultaneously steeped in the same reaction mixture, the relative intensity of remaining DHA-PC/Et decreased to 10.2%. Thus, the lower the embryonic MC, the greater was the changes in relative intensity of DHA-PC/Et. If moisture content was kept at 50.6% with the same embryonic mass (100 mg), the relative intensities of the remaining DHA-PC/Et also declined with reaction time (Fig. 5). For example, after 20 h interaction, the relative intensity decreased from the initial 67.4% to 9.0%.

When the reaction time (10 h) as well as the embryonic MC was fixed (e.g., 36.7%), the relative intensity of the peak (*m/z* 834.6) of remaining DHA-PC/Et was highly dependent on the mass of the desiccated embryos (Fig. 6). The difference in relative intensities between the initial relative intensity of 69.0% and the subsequent 26.3% showed that the amount (expressed by embryonic mass) had to be increased from 2.9 mg to 26.2 mg under other constant conditions.

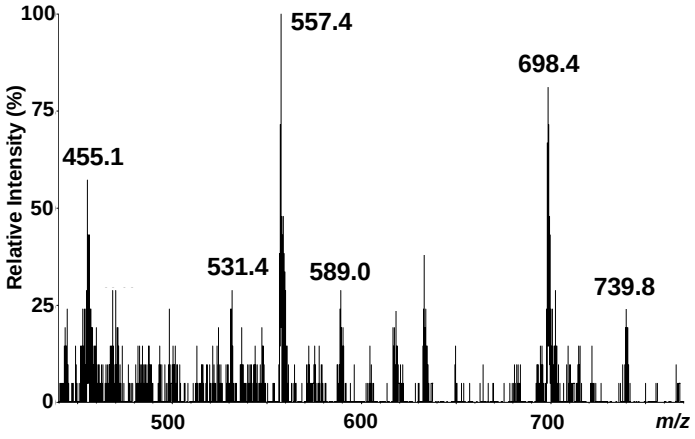

Fig. 3. ESI MS spectra of reaction mixtures from the reaction of LA-PC/D_3 **2**, with *Quercus glauca* seeds dried for 9 days.

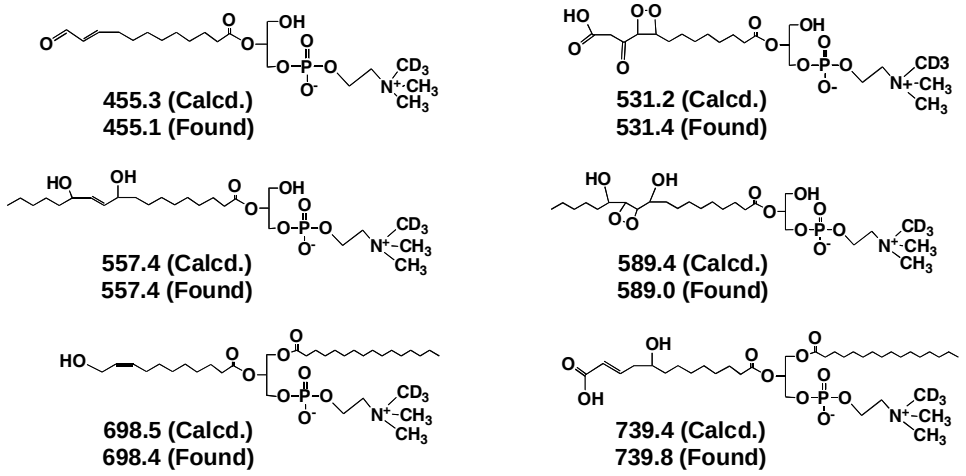

Fig. 4. Presumed structure of degradation products produced from LA-PC/D_3 **2** by the reaction with *Quercus glauca* seeds dried for 9 days.

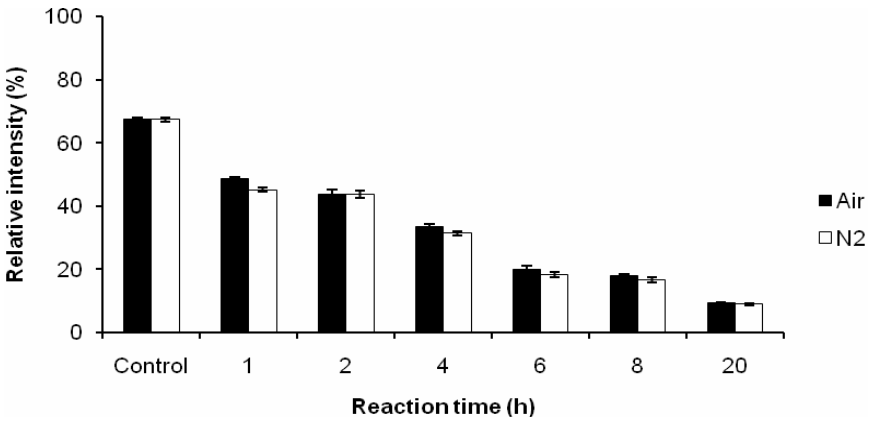

Fig. 5. Changes of relative intensity for the remaining DHA-PC/Et **3** after mixed with embryos having 50.6% MC.

DISCUSSION

Desiccation tolerance is one of the fundamental properties of orthodox seeds. Although it is an oversimplification, desiccation sensitivity is generally accepted as the obvious feature identifying recalcitrant seeds. In the present study, the loss of viability of mature seeds of blue Japanese oak obviously occurred after slow desiccation (Table 1). At the same time, lipid peroxidation measured as the amounts of TBARS significantly increased about three fold from the initial level of 42 nmol g^{-1} dwt to a maximum of 123 nmol g^{-1} dwt (by 17.6% MC) and then decreased to 61 nmol g^{-1} dwt. This clearly indicates that *Q. glauca* seeds were experiencing oxidative stress during drying. In the process of water loss, lipids localized to cellular membranes are exposed to free radicals which participate in some oxidative chemical reactions involved in lipid peroxidation [8, 9]. For example, in the axes of *Quercus robur*, the accumulation of a stable free radical and associated lipid peroxidation during drying appeared to be closely related [6]. In this study, we used our recent analytical technique [16] to deduce the possible structural changes of the embryo phospholipids resulting from oxidative degradation. The technique uses phospholipid molecular probes, LA-PC/D_3 and DHA-PC/Et in the precursor ion scan mode in tandem ESI MS spectrometry.

After reaction of LA-PC/D_3 with the dried embryo for 2 h, the extracted lipid fraction was analyzed with tandem ESI MS in precursor ion scan mode at *m/z* 187 instead of 184 for natural phosphatidylcholines in the embryo. If LA-PC/D_3 showed no oxidative degradation in this reaction at the polyunsaturated linoleoyl group, only a single signal should have been observed at *m/z* 761.7, which is a calculated *m/z* for the protonated form of LA-PC/D_3. However, a number of signals were found (Fig. 3). The chemical structure of the degradation products was deduced from their *m/z* and the well-documented mechanism in radical-initiated oxidative reaction of the polyunsaturated fatty acyl group [17, 18, 19, 20]. Fig. 4 shows some of their structures. The formation of these compounds from the molecular probe LA-PC/D_3 suggested that, polyunsaturated acyl groups in natural phospholipids **1** in the embryos may undergo oxidation during drying. The process may involve some reactive oxygen species (ROS) that are known to be produced in embryos by drying [8]. The signal pattern in the ESI MS spectrum of the lipid fraction extracted from the reaction mixture was inconsistent when the drying time differed, and consequently it was difficult to correlate the spectrometric data to viability loss in the embryo during drying. Therefore, we used another molecular probe DHA-PC/Et. This molecule bears docosahexaenoic acid (DHA) instead of linoleic acid (LA) and an ethyl group instead of CH_3 in **1** or CD_3 in **2**. DHA is known to be very sensitive to oxidation in hydrophobic environment. The difference in molecular mass between CH_3 and an ethyl group, CH_2CH_3, is 14, which is much larger than **3** between CH_3 and CD_3. Although DHA is not common in plants, we used DHA-PC/Et as a second molecular probe to investigate the relationship between phospholipid oxidation and viability loss of the dried embryo.

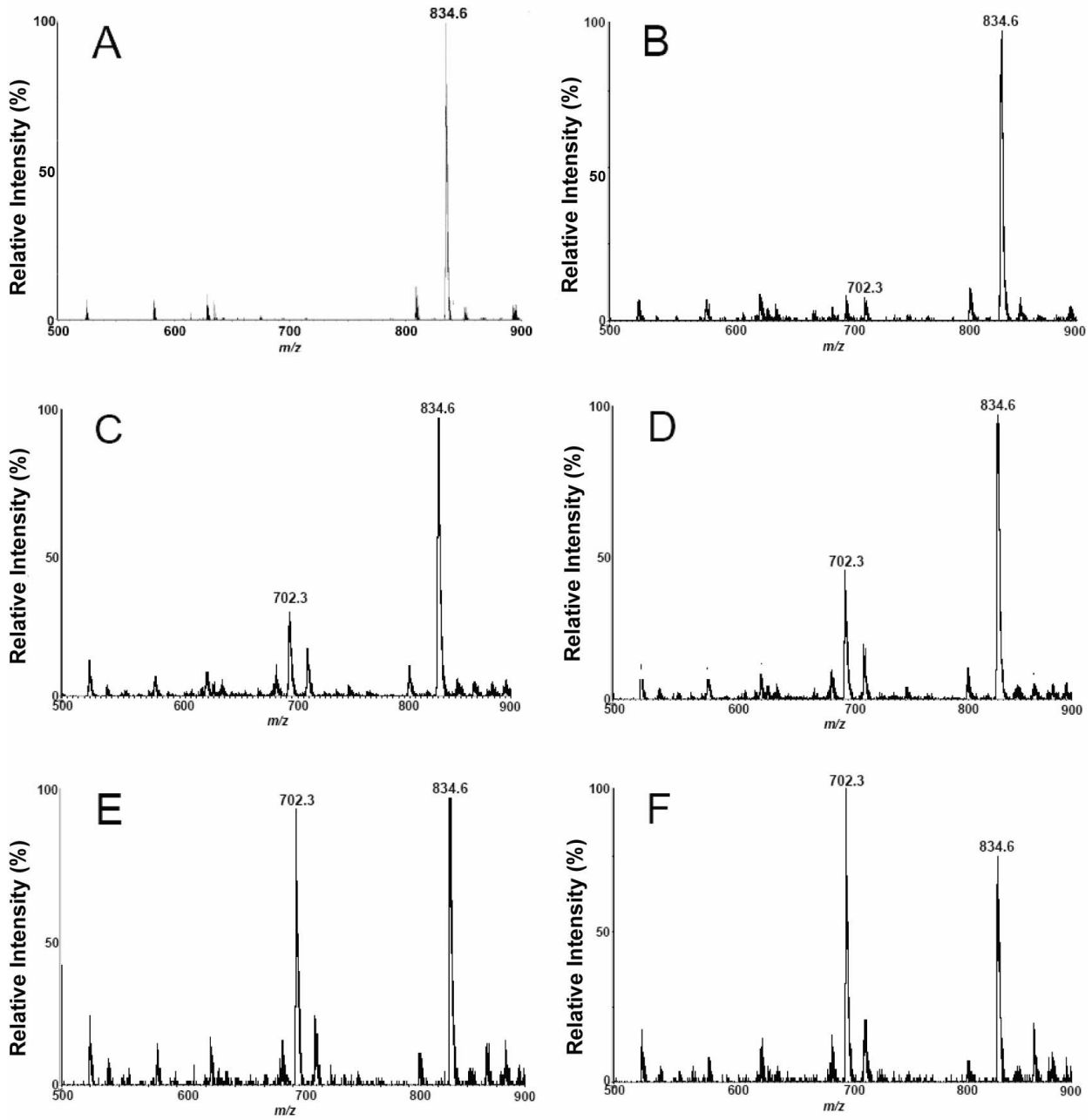

Fig. 6. ESI MS spectra of reaction mixtures from the reaction of DHA-PC/Et with different amounts of *Quercus glauca* seeds dried for 9 days. A: Control; B: 2.9 mg; C: 9.1 mg; D: 11.8 mg; E: 17.5 mg, and F: 26.2 mg.

The analysis of degradation products from DHA-PC/Et measured by precursor ion scan mode in tandem ESI MS, demonstrated that DHA-PC/Et was altered at the site of poly-olefinic structure (Fig. 7A) in *Q. glauca* seeds. This resulted in a mixture of degradation products that are shown in ESI MS spectra (Fig. 6, A-F). These products have a lower mass number than that of DHA-PC/Et (*m/z* 834.6 which coincided with the calculated exact mass of protonated **3**, 834.6). By increasing relative amount of the embryo to that of the molecular probe, a remarkable increase in relative intensity of a peak at *m/z* 702.3 compared to that of *m/z* 834.6 (DHA-PC/Et) was observed as seen in each spectrum A-F in Fig. 6. This clearly indicated that degradation of DHA-PC/Et to a

smaller degradation product with *m/z* 702.3 could be ascribed to some action of the embryo on DHA-PC/Et. The extent of the decomposition was found to be strongly dependent on the drying time of the seeds (Table 1). During viability loss resulting from slow desiccation, the relative intensity (%) of a peak at *m/z* 834.6 of the remaining DHA-PC/Et decreased sharply, suggesting that lipid decomposition might participate in the viability loss. This outcome suggested that naturally existing phospholipids in the embryo were also altered during drying, which would cause interference to the normal structure of cell membrane bilayer and death of seeds.

The intensity of the peak of the remaining intact DHA-PC/Et at *m/z* 834.6 relative to the total intensity of each peak of decomposition products was found to be significantly dependent on the exposure time of DHA-PC/Et to embryos in both nitrogen and air (Fig. 5). After 20 h reaction time (i.e., exposing time), the intensity of the peak at *m/z* 834.6 relative to the total decreased near to 10%. One important result in the data was that the difference in the decrease was slight but not significantly different in the nitrogen (N_2) and air atmospheres (Fig. 5). This similarity unambiguously indicated that the oxygen molecules (O_2) in the air were not involved in the decomposition of DHA-PC/Et suggesting that the embryos themselves were responsible for DHA-PC/Et decomposition. Therefore, the decomposition may be caused by some reactive oxygen species (ROS) as it is well known that ROS formation, during desiccation induces oxidative decomposition of the seed lipid affording malon dialdehyde (MDA) as a final decomposition product during induced loss of the viability [8, 10]. Similarly, Pukacka investigated the changes of the membrane phospholipid composition (e.g., PC, PE, and PI) in *Acer platanoides* and *Acer pseudoplatanus* using one dimensional thin layer chromatography, and found that maintenance of membrane phospholipid composition was involved in desiccation tolerance during maturation of *A. platanoides* seeds [21]. Moreover, enzyme assays of some antioxidative enzymes such as superoxide dismutase, catalase and ascorbate peroxidase to quench ROS during drying have been increasingly reported [8, 10, 11, 16, 22]. Phospholipids with polyunsaturated fatty acyl group are also well-documented to cause oxidative decomposition

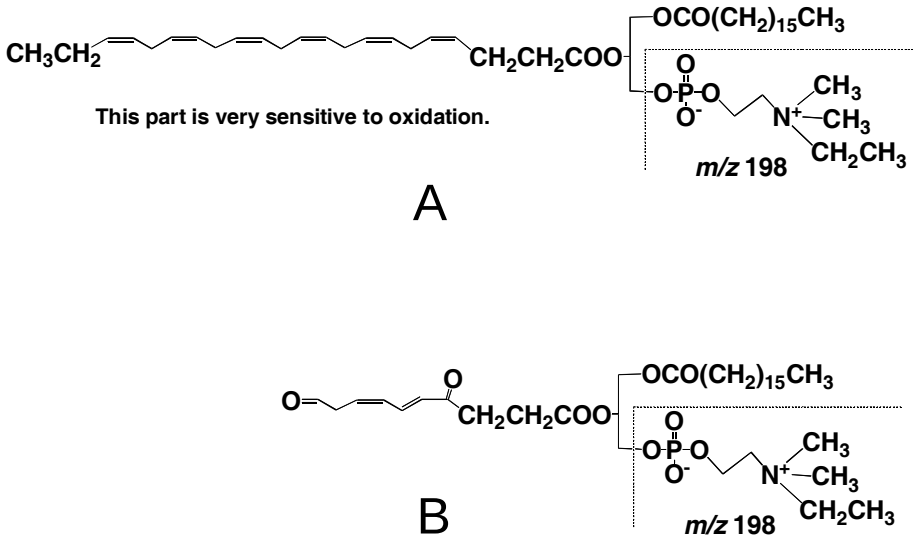

Fig. 7. A: Structure of 2-Docosahexaenoyl-1-heptadecanoyl-*sn*-glycero-phosphocholine (DHA-PC/Et **3**) (*m/z* 834.6; calculated exact mass of the protonated molecular species is *m/z* 834.6). This compound and its degradation product produced from the oxidation-sensitive site can be detected sensitively and selectively even in the presence of phospholipids in the embryo by precursor ion scan mode in tandem ESI MS using *m/z* 198 as a product ion. **B:** A presumed structure of the major decomposition product formed from DHA-PC/Et (*m/z* 702.3; calculated exact mass of the protonated molecular species is *m/z* 703.4).

affording decomposition products with lower molecular weight [20]. Based on related studies and the mechanism of lipid peroxidation reported so far [20, 23], we predicted that a major decomposition product showing a peak at *m/z* 702.6 in Fig. 6, B-F, has a molecular structure shown in Fig.7B.

This study demonstrated that, during drying bringing about viability loss of seeds of *Q. glauca* sensitive to drying, phospholipid degradation was directly detected for the first time by a new technique consisting of a molecular probe of DHA-PC/Et and tandem ESI MS spectrometry. This oxidation appeared to be caused by ROS formed in the embryos during drying rather than by oxygen molecules in the air. Also the extent of its degradation was clearly shown to be strongly dependent on the drying time of seeds, exposure time of the phospholipid and the amount of embryos used (i.e., indirect measure of ROS). These causal correlations indicate that stress of drying led to viability loss via ROS-mediated cell membrane damage in desiccation-sensitive seeds. Furthermore, the analytical method of this study showed possible structures of phospholipid degradation products which might show potential biological activity and, therefore, it will be useful in the investigations on the role of membrane and functional phospholipids in plant seed biology. Studies of plant seed biology have become increasingly important in the conservation of plant resources such as food and biofuel materials as well as global environments.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

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