Changes in membrane lipid composition play multiple roles in plant adaptation and survival in the face of chilling and freezing damage. An electrospray ionization tandem mass spectrometry (ESI-MS/MS)-based approach has been used to quantitatively profile membrane lipid molecular species in plant response to low temperatures. This method involves the direct infusion of unfractionated lipid extracts into a mass spectrometer in the precursor and neutral loss scanning modes to identify and quantify lipid species. The profiling analysis reveals significant and distinct lipid changes during cold acclimation and freezing. Comparative profiling of wildtype and mutants provides information about the metabolic and cellular functions of specific phospholipase D genes and enzymes.

Introduction
Membrane lipids undergo substantial alterations when plants are exposed to low temperatures. The degree of fatty acid unsaturation and the content of phospholipids increase during cold acclimation. Such lipid changes enhance membrane fluidity and reduce the propensity of cellular membranes to undergo freezing-induced non-bilayer phase formation, thus enhancing membrane integrity and cellular functions (Uemura et al. 1995). In addition, membrane lipids are rich sources for generating lipid mediators. Lipid mediators, such as phosphatic acid, lysophospholipids, and free fatty acids, are involved in various cellular processes, including signal transduction, vesicular trafficking, and/or cytoskeletal reorganization, in plant stress responses (Wang 2004). Thus, membrane lipid changes play both structural and regulatory roles in plant adaptation and survival when subjected to low-temperature damage. Information on how lipid species change and how the alterations are generated will help us understand the functions of lipids and membranes in plant response to temperature stresses.

Analysis of membrane lipid species has been technically challenging, as lipids comprise diverse molecular species. Glycerolipids can be classified into head-group classes with each class composed of various molecular species, as the fatty acids or other hydrocarbon portions vary in chain length and unsaturation. Recently, lipiddomics has emerged as a powerful strategy to fully characterize lipid molecular species and their biological roles with respect to proteins and genes. Approaches based on electrospray ionization tandem mass spectrometry (ESI-MS/MS) have been developed to comprehensively analyze lipid composition in animals, yeast, and plants (Brügger et al. 1997, Welti et al. 2002, Welti and Wang 2004, Han and Gross 2005, Wenk 2005). Here, we describe an ESI-MS/MS-based platform for

Abbreviations – DGDG, digalactosyldiacylglycerol; ESI-MS/MS, electrospray ionization tandem mass spectrometry; MGDG, monogalactosyldiacylglycerol; PA, phosphatic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLA, phospholipase; PLC, phospholipase C; PLD, phospholipase D; PS, phosphatidylserine; SQDG, sulfoquinovosyldiacylglycerol
quantitative profiling of membrane lipid species and also its application to study changes of lipid molecular species and metabolic and cellular functions of specific phospholipases in *Arabidopsis* responses to cold acclimation and freezing.

**ESI-MS/MS-based profiling of lipid molecular species**

ESI-MS/MS-based lipid profiling utilizes a triple quadrupole tandem mass spectrometer and the general procedure outlined in Fig. 1 (Welti et al. 2002). An unfractionated lipid extract is introduced by continuous infusion into the ESI source. Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head-group fragment (Table 1). Lipid species within each head group are identified in terms of total carbon number and total double bonds. The lists of detected masses and peak areas are automatically sorted against lists of the masses of lipid species. The signal for each molecular species is corrected for isotopic overlap of the lipid species with other species (Welti et al. 2002) and then compared in magnitude with the signals of two internal standards of each head-group class. This method produces good precision and allows detection of small changes in lipid composition.

**Fig. 1.** Process of lipid profiling using ESI-MS/MS. Lipids are extracted from plant tissues by solvents. Special caution is needed to inactivate lipolytic enzymes. In particular, PLD is readily activated to produce PA; the current method to minimize PLD activation is to immerse tissue rapidly into hot isopropanol to inactivate the enzyme. Lipid species within each head group are identified by total carbon number and total double bonds. Molecular species of each head class are quantified by comparing with the signals of the internal standards. ESI-MS/MS, electrospray ionization tandem mass spectrometry; PA, phosphatidic acid; PLD, phospholipase D.
Currently, this methodology allows routine analysis of approximately 200 polar plant lipid molecular species in nine head-group classes, which include all of the major membrane glycerolipids (Table 1). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are analyzed as singly charged positive \([M+H]^+\) ions, phosphatidylglycerol (PG), phosphatidylinositol (Pl), phosphatidic acid (PA), phosphatidylserine (PS), and sulfoquinovosyldiacylglycerol (SQDG) are analyzed as singly charged negative \([M-H]^-\) ions, and monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are analyzed as singly charged positive \([M+Na]^+\) ions (Table 1). This approach can detect minor molecular species, such as PA and lysophospholipids, as well as minor PE and PC species, containing long-chain fatty acyl groups. Only simple sample preparation and small samples are needed in order to identify and quantify these membrane structural glycerolipid species and metabolites. A routine analysis provides information on phospholipids and glycolipids speculated to the level of head group and number of carbon atoms and number of double bonds present in the acyl chains. Individual acyl species can be determined separately via product ion analysis of the molecular ions in the negative mode (Welti et al. 2002, 2003). Such analysis allows tentative assignment of the acyl product that produces the larger signal to the 2-position in a diacyl phospholipid (Murphy 1993, Welti et al. 2002).

### Profiling changes in lipid molecular species during cold acclimation and freezing

Lipid profiling has been applied to analyze changes in lipid molecular species of Arabidopsis under low-temperature stresses. Significant changes in membrane lipid molecular species occurred when Arabidopsis plants were cold-acclimated at 4 ºC (Welti et al. 2002). PC, PE, PG, MGDG, and DGDG species that contain two polyunsaturated acyl species, such as 36:5- (18:3-18:2-) and 36:6- (di18:3-) PC, 36:5- and 36:6-PE, 36:6- (18:3-16:3) MGDG, and 36:6-DGDG, increased, while the levels of more saturated species in these lipid classes, such as 36:2- (in which 18:0-18:2- combinations are more common than 18:1-18:1-) and 36:3- (in which 18:1-18:2- combinations are more common than 18:0-18:3-) PC, decreased. By contrast, PI molecular species levels were unchanged. Cold acclimation also induced significant increases in species of lipid metabolites lysoPC, lysoPE, and PA, suggesting that phospholipases, such as phospholipase A (PLA) and phospholipase D (PLD), are activated during cold acclimation.

### Table 1. ESI-MS/MS analysis parameters (using Applied Biosystems API 4000) for plant lipids. DGDG, digalactosyldiacylglycerol; ESI-MS/MS, electrospray ionization tandem mass spectrometry; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SQDG, sulfoquinovosyldiacylglycerol.

<table>
<thead>
<tr>
<th>Lipid(s) analyzed</th>
<th>Ion analyzed</th>
<th>Scan mode</th>
<th>Polarity</th>
<th>Collision energy (V)</th>
<th>Time (minutes)</th>
<th>Species identified (total acyl carbon : total double bonds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC/lysoPC</td>
<td>[M+H]^+</td>
<td>Precursors of 184^+</td>
<td>+</td>
<td>40</td>
<td>1.5</td>
<td>PC = 32:0; 34:2;3:4; 36:1;2;3:4:5:6; 38:2;3:4:5; 40:2;3:4:5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LysoPC = 16:0;1; 18:0;1;2;3</td>
</tr>
<tr>
<td>PE/lysoPE</td>
<td>[M+H]^+</td>
<td>Neutral loss of 141</td>
<td>+</td>
<td>28</td>
<td>3</td>
<td>PE = 34:1,2;3,4; 36:1,2;3,4,5:6; 38:2,3:4,5:6; 40:2,3; 42:2,3,4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LysoPE = 16:0;1; 18:1;2;3</td>
</tr>
<tr>
<td>PA/PG/lysoPG</td>
<td>[M–H]^-</td>
<td>Precursors of 153^-</td>
<td>–</td>
<td>-57</td>
<td>4</td>
<td>PA = 34:1;2;3,4:6; 36:2,3:4,5:6; PG = 32:0;1; 34:0;1;2;3,4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LysoPG = 16:0;1; 18:1;2;3</td>
</tr>
<tr>
<td>PI</td>
<td>[M–H]^-</td>
<td>Neutral loss of 87</td>
<td>–</td>
<td>-34</td>
<td>4</td>
<td>34:1,2;3,4; 36:1,2;3,4:5:6</td>
</tr>
<tr>
<td>PS</td>
<td>[M–H]^-</td>
<td>Neutral loss of 87</td>
<td>–</td>
<td>-60</td>
<td>4</td>
<td>32:0; 34:3; 36:6</td>
</tr>
<tr>
<td>SQDG</td>
<td>[M–H]^-</td>
<td>Precursors of 225^-</td>
<td>–</td>
<td>-50</td>
<td>5</td>
<td>34:1,2;3,4,5:6; 36:1,2;3,4,5:6; 38:3,4,5:6</td>
</tr>
<tr>
<td>MGDG</td>
<td>[M+Na]^+</td>
<td>Precursors of 243^+</td>
<td>+</td>
<td>84</td>
<td>5</td>
<td>34:1,2;3,4,5:6; 36:1,2;3,4,5:6; 38:3,4,5:6</td>
</tr>
<tr>
<td>DGDG</td>
<td>[M+Na]^+</td>
<td>Precursors of 243^+</td>
<td>+</td>
<td>84</td>
<td>5</td>
<td>34:1,2;3,4,5:6; 36:1,2;3,4,5:6; 38:3,4,5:6</td>
</tr>
<tr>
<td><strong>Total time</strong></td>
<td>Inc. washes and sample changes</td>
<td></td>
<td></td>
<td>55</td>
<td></td>
<td></td>
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</table>

Physiol. Plant. 126, 2006
Freezing induced a decrease in total membrane lipid content, and the decrease resulted primarily from the loss of PC, PE, and PG (Welti et al. 2002). At −8 °C, a sublethal freezing temperature for cold-acclimated Arabidopsis, the amount of almost all molecular species of PG, PC, and PE decreased, with the 36:4 and 36:5 species contributing the most to the decline in PE and PC levels. The levels of lipid metabolites PA, lysoPC, and lysoPE increased dramatically. As during cold acclimation, the change in PI molecular species was minimal, and some PI species actually increased. MGDG levels tended to decline, but no net loss of DGDG occurred in Arabidopsis exposed to −8 °C.

**Insights into the metabolic processes that generate lipid changes**

The profiling data provide clues as to the metabolic processes underlying the lipid changes. The elevation of the amount of unsaturated fatty acids and most glycerolipids in cold acclimation, which is consistent with earlier observations (Uemura et al. 1995, Thomashow 1999, Iba 2002), indicate an increase in fatty acid desaturation and membrane lipid biosynthesis. Lipid profiling provided new information about the identities of the polar lipid molecular species that were changed during cold acclimation (Welti et al. 2002). Quantification of the lipid metabolites, PA and lysophospholipid species, suggests that the activity of specific types of lipolytic enzymes increases during cold treatments. The freezing-induced increase in PA and lysophospholipids are several fold greater than that induced by cold acclimation. This indicates that lipid-hydrolytic activities are elevated much more in freezing than in cold acclimation.

In addition, the detailed ESI-MS/MS-based analysis provides information as to whether any specific lipid molecular species are preferentially metabolized and whether particular reactions occur. Despite the general decline of glycerolipids during freezing, mole percentages of 34:2 PC and 34:2 PE species are higher in the −8 °C-treated plants than in the cold-acclimated plants, suggesting that these species, with an acyl composition of 16:0-18:2, are somewhat resistant to hydrolysis (Welti et al. 2002). This might result from a preference of a hydrolytic enzyme(s) for more unsaturated species or partial inaccessibility of these lipid species to hydrolytic enzymes, perhaps due to an asymmetric distribution of acyl species across membrane lipid bilayers, but this is difficult to determine.

In plants, several lipolytic enzymatic activities have been described; these include PLD, PLA, phospholipase C (PLC), non-specific acyl hydrolase, and galactolipases. PLD and PLC have been implicated in plant response to low temperatures (Ruelland et al. 2002, Welti et al. 2002, Li et al. 2004). The large decline in major membrane phospholipids, but not galactolipids, suggests that at the sublethal freezing temperature, phospholipases are activated to a greater extent than galactolipases. The low level of the PLA products, lysophospholipid, in comparison with the PLD product, PA, indicates that PLD is more active than PLA in plant tissues. In addition, comparison of the molecular species profile of PA with other lipid classes helps identify the substrates that give rise to PA. Upon freezing, most of the PA species that increased have the same acyl chains as the PC species that decreased (Fig. 2). These data, together with the analysis of PLD1-deficient mutant (described in the following section), lead to the conclusion that freezing-induced PA is derived primarily from PC. However, upon freezing, plants gained one PA molecular species, 34:6 PA, that was absent from the plants before the freezing treatment. Moreover, 34:6 PA has the acyl composition, 18:3-16:3, which is found otherwise only in galactolipids, particularly in MGDG (Fig. 2). This suggests that some PA is formed by phosphorylation of diacylglycerol produced by hydrolysis of MGDG.

**Comparative profiling to tease out the metabolic and cellular functions of PLDs**

One major lipid change in plant response to low temperatures is the increase in PA molecular species. Some PA species could be direct products of PLD, a major family of phospholipases in plants; Arabidopsis has 12 PLD genes that are classified into six types, PLDα1(3), β2, γ3, δ, ε, and ζ(2) (Wang 2005). Lipid profiling, combined with genetic manipulation, has proved effective to determine the role of specific PLDs in the freezing-induced lipid hydrolysis. Compared with wildtype plants after freezing, PLD1-deficient plants had a higher level of PC and a lower level of PA (Fig. 2), whereas PE and PG levels declined to a similar extent in wildtype and PLDα-deficient plants (Welti et al. 2002). The lack of difference between wildtype and mutant plants in the level of PE and PG suggests that other enzymes are responsible for the freezing-induced hydrolysis of PE and most of the PG species. The difference in PC between wildtype and PLDα-deficient plants indicates that PC is the major in vivo substrate for PLDα during freezing-induced activation (Fig. 2). This preference of PLDα for PC is supported by in vitro data (Pappan et al. 1998). PLD1 is responsible for approximately half of PC hydrolyzed and about half of the PA produced under the freezing conditions tested.

By comparison, genetic manipulation of PLDδ has only subtle effects on membrane lipid composition.
Fig. 2. Freezing-induced changes in PA, PC, and MGDG in wildtype and PLDα1-deficient Arabidopsis as revealed by ESI-MS/MS (Welti et al. 2002). Pre-flowering plants were cold-acclimated at 4 °C for 3 days (solid bar) and then subjected to a temperature drop from 4 to –2 °C at 3 °C per hour. When the temperature reached –2 °C, ice crystals were placed on the soil to induce crystallization and prevent supercooling. After 2 h at –2 °C, the temperature was lowered to –8 °C at 1 °C per hour. After 2 h at –8 °C, plants were harvested for lipid analysis (hatched bar). Values are mean ± so (n = 4 or 5). ESI-MS/MS, electrospray ionization tandem mass spectrometry; PA, phosphatidic acid; PC, phosphatidylcholine; MGDG, monogalactosyldiacylglycerol; PLD, phospholipase D.

(Li et al. 2004). Upon freezing, the amount of PA in PLDδ-null and PLDδ-overexpressing plants is about 80 and 125%, respectively, of wildtype. Increased PLDδ expression leads to the production of more polyunsaturated PA species. The results suggest that in contrast to PLDα-deficient plants, the activity of PLDδ does not contribute to substantial lipid hydrolysis.

These metabolic differences in lipid changes indicate different functions of PLDα1 and PLDδ in freezing. Indeed, Arabidopsis deficient in PLDα1 displays an opposite phenotype in freezing tolerance compared to Arabidopsis deficient in PLDδ (Welti et al. 2002, Li et al. 2004). Antisense suppression of the most abundant plant PLD, PLDα1, rendered Arabidopsis plants more tolerant to freezing. During freezing, the total amounts of PA increased more than five-fold in wildtype, but PLDα1-deficient plants accumulated only 48% as much PA as wildtype plants (Fig. 2). The increase in PA is accompanied by a decrease in PC. PC is a bilayer-stabilizing lipid, whereas PA has tendency to form hexagonal II phase in the presence of cations. The propensity of membranes to form the hexagonal phase has been suggested to be a key event in freezing injury. Thus, suppression of PLDα1 may decrease the propensity of membrane lipids to undergo a transition from lamellar to hexagonal II phase, thus increasing freezing tolerance (Welti et al. 2002).

By contrast, gene knockout of the plasma membrane-associated PLDδ rendered Arabidopsis plants more sensitive to freezing, whereas overexpression of PLDδ increased freezing tolerance (Li et al. 2004). These results indicate that PLDδ positively affects the cellular response to freezing. Lipid profiling data indicate that PLDδ is not involved in the major freezing-induced decline of membrane lipids; rather, it produces a small increase in selective PA species. Thus, while high PLDα1 activity destabilizes membranes and increases membrane leakage, regulated increase of PLDδ may produce signaling PA species that mitigate stress damage. Specifically, PLDδ and the resulting PA decrease cell death promoted by the reactive oxygen species H₂O₂. The level of H₂O₂ increases in plant cells in response to freezing stress. Thus, the impaired response to oxidative stress in PLDδ-null plants may be a basis for the decreased freezing tolerance. It has been proposed that PLDδ positively mediates plant freezing tolerance, through its role in signaling to enhance resistance to damage inflicted by reactive oxygen species (Zhang et al. 2003, Li et al. 2004).
Biochemical and molecular analyses of PLDα1 and PLDδ have provided valuable insights into the question of how the two PLDs can have different functions. First, PLDα1 and PLDδ are regulated differently, as indicated by their different requirements for Ca\(^{2+}\) and free fatty acids; PLDδ, but not PLDα1, is stimulated by oleic acid (Wang and Wang 2001). Second, the membrane association of the two PLDs is different. PLDδ is tightly associated with the plasma membrane and with microtubule cytoskeleton, whereas PLDα1 is present in the plasma membrane and intracellular membranes, as well as soluble fractions (Gardiner et al. 2001, Wang and Wang 2001). Third, PLDα1 and PLDδ have distinctively different substrate preferences. PLDα1 prefers PC to PE, and profiling lipid molecular species indicates that PLDα1 uses primarily PC in the cells during freezing stress. On the other hand, surface-dilution kinetics analysis shows that PLDδ prefers PE 10-fold to PC (Qin et al. 2002). Fourth, the two PLDs are expressed differently. Whereas PLDα1 is expressed highly in young leaves, the mRNA levels of PLDδ increase in senescent leaves and in response to extreme stresses such as dehydration and high salt stress (Katagiri et al. 2001, Wang and Wang 2001). These distinguishable properties indicate that PLDα1 and PLDδ are activated differently by Ca\(^{2+}\), PIP\(_2\), FFA, and membrane lipid environments and that their activation can lead to the selective hydrolysis of phospholipids (e.g. PC vs. PE) and the production of distinct pools of PA. The activation of PLDα1 and PLDδ can result in the production of PA in different location, timing, amounts, and molecular species, thus giving rise to unique metabolic and physiological functions.

**Perspectives and challenges**

Quantitative and comprehensive lipid profiling is an emerging technology. As described above, the lipidomics approach has demonstrated great potential to facilitate understanding the functioning of membrane lipids in plant adaptation and tolerance to low-temperature stresses. It has helped to characterize (i) lipid metabolic pathways involved in low-temperature responses, (ii) roles of specific genes/enzymes in the stresses, and (iii) lipid species that serve as substrates and products of specific enzymes. In addition, profiling information can be used for gene discovery by aiding identification of genes in mutants. ESI-MS/MS-based lipid profiling is inclusive for most lipid molecular species. It should be possible to extend the ESI-MS/MS platform so that routine lipid profiling will cover additional lipids, such as sphingolipids, phosphoinositides, N-acyl phosphatidylycerol, and free fatty acids.

However, much needs to be overcome to achieve true comprehensiveness and robustness in the analyses (Welti and Wang 2004). For example, the ESI-MS/MS-based lipid profiling presented here is a targeted strategy and, thus, such analysis cannot detect the presence of lipids in untargeted classes. In addition, profiling lipids in subcellular membranes has been challenging, because lipolytic enzymatic activity could result in alteration of lipid composition during homogenization and subcellular fractionation. Furthermore, a full realization of the potential of lipid profiling will require development of better informatics for efficient data handling and analysis.

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