N-Acylphosphatidylethanolamine in Dry and Imbibing Cottonseeds[1]

Amounts, Molecular Species, and Enzymatic Synthesis

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N-Acylphosphatidylethanolamine (NAPE), an unusual acylated derivative of phosphatidylethanolamine (PE), was recently shown to be synthesized from PE and free fatty acids in cotyledons of cotton (*Gossypium hirsutum* L.) seedlings (K.D. Chapman, T.S. Moore [1993] Plant Physiol 102: 761–769). Here we report that NAPE is present in dry seeds of cotton and increases with time of imbibition from 2.31 nmol/seed in dry seeds to 4.26 nmol/seed in 4-h-soaked seeds. Total phospholipid/seed also increased such that the relative percentage of NAPE was similar in dry and soaked seeds (2.3 mol% compared to 2.6 mol%, respectively). The major molecular species of NAPE were identified in both dry and soaked seeds by fast atom bombardment mass spectrometry and collisionally activated dissociation tandem mass spectrometry as 16:0/18:2-PE(N-palmitoyl), 16:0/18:2-PE(N-linoleoyl), and 18:2/18:2-PE(N-palmitoyl). The specific activity of NAPE synthase in seed extracts increased with increasing time of imbibition from 35 pmol h^{-1} mg^{-1} protein in dry seeds to 129 pmol h^{-1} mg^{-1} protein in 4-h-soaked seeds. Collectively, our results indicate that NAPE is present in dry cottonseeds and synthesized during imbibition. The biosynthesis of NAPE provides a mechanism for maintaining membrane integrity during seed rehydration and may indicate that NAPE plays a protective role in intracellular membranes of plant tissues, as has been suggested for intracellular membranes of animal tissues.

NAPE, which was first identified as a minor constituent of wheat flour (Bomstein, 1965), has recently become recognized as a widespread, albeit minor, phospholipid component in intracellular membranes of plants (Chapman and Moore, 1993a). NAPE is synthesized in vivo under normal physiological growth conditions in cotyledons of cotton (*Gossypium hirsutum* L.) seedlings. The biosynthesis of NAPE appears to occur by direct acylation of PE with FFA (Chapman and Moore, 1993a, 1993b; Chapman et al., 1995b).

Although the function of NAPE is not known, it accumulates in animal tissues under pathological conditions that involve the degradation of intracellular membranes (e.g. ischemic brain tissue [Natarajan et al., 1985]; infarcted heart tissue [Epps et al., 1979, 1980]; and degenerating epidermal cells [Gray, 1976]). Biophysical studies show that NAPE helps to stabilize a bilayer configuration in membranes (LaFrance et al., 1990; Schmid et al., 1990) and to reduce the permeability of model membranes to ions (Domingo et al., 1993). LaFrance et al. (1990) reported that the acyl chain length on the ethanolamine headgroup structure of NAPE may contribute to the stabilization effect. When the N-acyl chain contained 10 or more carbon atoms, it penetrated into the bilayer, whereas it remained at the level of glycerol for shorter N-acyl chains. The stabilizing function of NAPE is attributed to the higher degree of hydration at the bilayer surface (caused by the disruption of the intermolecular hydrogen bonds between the phosphate and amino groups) and immobilization of the lipid molecule by the third acyl chain (LaFrance et al., 1990). Hence, it was postulated that NAPE is synthesized in membranes of damaged tissues to help maintain cellular compartmentalization (Schmid et al., 1990). Recent support for this hypothesis has come from studies of the permeability properties of NAPE-containing liposomes (Domingo et al., 1993): increasing the NAPE content of PC liposomes resulted in decreased leakage of K$^+$ and carboxyfluorescein.

Environmental stresses exist that can alter the integrity of the lipid bilayer of plant cell membranes. These include desiccation (Senaratna et al., 1984), freezing (Borochov et

Abbreviations: CAD, collisionally activated dissociation; FAB, fast atom bombardment; FFA, free fatty acid(s); H$_{II}$, hexagonal II; MS/MS, tandem MS; NAPE, N-acylphosphatidylethanolamine(s); PC, phosphatidylcholine(s); PE, phosphatidylethanolamine(s); PEHA, pentaethylenehexamine.

al., 1987), and anoxia (McKersie et al., 1988). McKersie and co-workers have observed that the physical changes associated with membrane stress are due in part to FFA accumulation (McKersie, 1991). Recently, van Bilsen et al. (1994) demonstrated that an increase in FFA not only increased the T_m of membranes isolated from aging pollen that had imbibed, but also disrupted the membrane bilayer, causing extensive leakage of cellular solutes. Seed imbibition, like imbibition by pollen, subjects dry membranes to destructive forces (osmotic pressures) and therefore could result in the disruption of the phospholipid bilayer (Crowe et al., 1989a, 1989b). Since these cellular stressors can result in a loss of membrane integrity and even death of the organism (Simon, 1978), plants likely must be able to stabilize their intracellular membranes during seed imbibition.

The focus of this study was to determine the amounts of NAPE and NAPE synthase (the enzyme that synthesizes NAPE from PE and FFA) activity in dry and imbibing cottonseeds. In addition, NAPE molecular species isolated from cottonseeds were identified by FAB ionization followed by MS/MS. Collectively, these results show that NAPE is present in dry seeds and increases concomitantly with NAPE synthase specific activity during seed imbibition (4 h). Biosynthesis of NAPE, in theory, stabilizes intracellular membranes by reducing the cellular concentration of FFA and by producing a phospholipid with membrane-stabilizing properties. Our data are consistent with the notion that NAPE is present and synthesized in cottonseed membranes, possibly to help protect them from intracellular damage during imbibition.

MATERIALS AND METHODS

Plant Material

Cottonseeds (*Gossypium hirsutum* L.) were surface sterilized in 20% bleach for 2 min and rinsed well with distilled water. Seeds were soaked with aeration for 4 h. Decoated dry seeds were utilized for the 0 time point. Four hours is sufficient imbibition time for reproducible germination of cottonseeds (greater than 95% germination after 24 h). Cottonseeds, cv Stoneville 7A glandless (1991 harvest), were a gift from Dr. R.B. Turley (U.S. Department of Agriculture-Agricultural Research Service, Cotton Physiology and Genetics, Stoneville, MS).

Lipid Extraction

At selected intervals, imbibing seeds were frozen in liquid nitrogen and powdered with a pestle in a chilled mortar. The material was added to boiling isopropanol (70°C) in the ratio of 0.5 parts to 2 parts (w/v) and heated for 30 min. After 1 min of cooling in ice, 1 part chloroform and 0.3 parts water (v/v) were added to the mixture and lipids were extracted at 4°C overnight. Isopropanol was substituted for methanol in lipid extractions as a precaution to avoid potential artifacts generated by phospholipases (DeLaRoche et al., 1973). Insoluble material was sedimented by centrifugation at 2000 rpm for 5 min. The supernatant was decanted and 1 part chloroform, 2 parts 1 M KCl were added to induce phase separation. The aqueous layer was aspirated off, and the organic layer was washed three times with 2 parts 1 M KCl. The resulting chloroform phase was collected and stored at −20°C under nitrogen gas.

Class Separation of Lipids

The total lipid extracts were separated into nonpolar lipids, glycolipids, and phospholipids over solid phase extraction cartridges (Whatman, Silica Gel G) by sequential elution with solvents (hexane:diethyl ether [4:1, v/v]; acetone:acetic acid [100:1, v/v]; and chloroform:methanol [2:1, v/v], respectively). Individual phospholipid classes were corrected for losses on solid phase extraction cartridges by multiplying by a conversion factor obtained with known amounts of commercially available standards subjected to the same elution conditions (e.g. PE conversion factor = 1.4; NAPE conversion factor = 1.07). Phospholipids were stored in chloroform:methanol (2:1, v/v) in glass vials at −20°C under nitrogen gas.

TLC

Phospholipids were separated by two-dimensional TLC (Whatman Silica Gel G-60 plates) in chloroform:methanol:7 M NH₄OH (65:35:4, v/v) for 55 min and then in chloroform:methanol:acetic acid:water (80:12.5:12.5:3, v/v) for 70 min (Nichols, 1964). Phospholipids were made visible by exposure to iodine vapors and quantified as phospholipid phosphate according to the method of Duck-Chong (1978). Phospholipid classes were identified by co-chromatography with authentic standards and spray reagents as previously described (Chapman and Moore, 1993a).

MS

For MS, phospholipids were extracted from approximately 10 g of cottonseeds (dry and soaked) as described above. Phospholipids were separated by one-dimensional TLC (Silica Gel G-60, 250-μm layer, Whatman) as previously described (Chapman and Moore, 1993a). Cottonseed NAPE and PE were extracted from silica gel by adding 6 mL of chloroform:methanol:water (1:2:0.5, v/v; overnight at 4°C). The silica was sedimented by centrifugation (15 min, 2000 rpm, Beckman TJ-6 centrifuge) and the supernatants were collected. Chloroform (2 mL) and 1 M KCl (4 mL) were added to the supernatants. The organic phase was collected and washed two times with 1 M KCl as above.

Purified PE and NAPE were analyzed by FAB-MS/MS. Mass spectra were recorded on a JEOL HX-110 double-focusing mass spectrometer operating in the negative ion mode. Ions were produced with a 6-kilo-electron volt beam of Xe atoms. The accelerating voltage was 10 kilo-electron volts and the resolution was set at 1000. The instrument scanned at a rate of 40 to 60 s from 100 to 1100 D. Approximately 1 to 5 μg of sample in 1 μL of chloroform:methanol (1:1, v/v) was mixed with 2 μL of the matrix PEHA or a 1:2 (v/v) mixture of PEHA with 15-crown-5 on the FAB probe-tip. FAB-CAD-MS/MS was employed using linked scanning at a constant magnetic field strength:electric sector

voltage ratio. Helium was used as the collision gas in a cell located in the first field-free region. The helium pressure was adjusted to reduce the peak height of the selected precursor ion by approximately 50%.

NAPE Synthase Assay

NAPE synthase activity was measured in homogenates of cottonseeds at the same stages as were used for lipid analysis. Seed homogenates were prepared by chopping decoated seeds with a single-edge razor blade in 100 mM phosphate (pH 7.2), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 400 mM Suc. Homogenates were filtered through four layers of presoaked (with homogenization medium) cheesecloth and used directly for enzyme assays and protein estimations. NAPE synthase assays were conducted by previously developed methods (Chapman and Moore, 1993b). Reactions were initiated by adding [1-^{14}C]palmitic acid (0.25 μCi [4.4 nmol] of [^{14}C]palmitic acid in 2.5 μL of ethanol) to 0.5 mL of homogenate and briefly vortexing. Assay reaction mixtures were incubated with shaking (120 oscillations min^{-1}) at 45°C (optimum temperature for enzyme activity in vitro; Chapman and Moore, 1993b) for 30 min. Reactions were stopped by the addition of 2 mL of hot propanol (70°C). The synthesis of [^{14}C]NAPE from [1-^{14}C]palmitic acid was assessed after extraction of lipids from the assay reaction mixtures and separation by TLC (Chapman and Moore, 1993b). Radioactive NAPE was identified and quantified by radiometric scanning (Bioscan [Washington, DC] system 200 imaging scanner) of TLC plates (Chapman et al., 1995b).

Protein content of homogenates was estimated by Coomassie blue dye-binding assays as previously described (Chapman and Moore, 1994), employing BSA as the standard.

RESULTS

Fresh weight and phospholipid content changed in cottonseeds during imbibition (Fig. 1). Cottonseeds approximately doubled in fresh weight after 4 h of imbibition (Fig. 1A). Quantification of phospholipids (Fig. 1B) on a fresh weight basis revealed a decline in phospholipid content during seed imbibition; however, on a per-seed basis, phospholipid content increased. This apparent discrepancy is due to the rapid uptake of water and indicates that phospholipid biosynthesis in cottonseeds may begin as early as 1 h after soaking.

Amounts of PE and NAPE per seed increased approximately 2-fold after 4 h of imbibition (Fig. 2). PE content increased from 19.8 nmol/seed to 39.9 nmol/seed; NAPE content increased from 2.31 nmol/seed to 4.26 nmol/seed. The rise in PE content preceded that for NAPE. PC, the major phospholipid in cottonseeds, also increased during seed imbibition (about 1.5-fold; not shown). The relative proportion of NAPE in dry and imbibing (4 h) seeds was similar (2.3 mol% compared to 2.6 mol% of total phospholipids, respectively).

Negative ion FAB mass spectra of NAPE isolated from dry and imbibing cottonseeds are shown in Figure 3. A

number of even mass ions corresponding to the [M − H]$^-$ ions of NAPE molecular species were evident in the high-mass regions of both spectra. Prominent peaks of fatty acid anions derived from the O-acyl groups were observed in the low-mass region (Fig. 3, A and B, insets) at m/z 255 (16:0), 279 (18:2), 297 (19:0), and 311 (20:0). Predictable mass shifts arising from N-acylation of the characteristic PE headgroup fragments (in unsubstituted PE, these are found at m/z 140, 180, and 196; see Gage et al., 1994) were found to occur and provided diagnostic ions for the N-substituted PE head groups (see Fig. 4). The fragmentation pattern rationalized above was fully supported by the MS/MS studies, which allowed the composition of individual molecular species to be characterized. For example, the CAD-MS/MS analysis of the [M − H]$^-$ ion at m/z 952 (Fig. 3C) revealed the presence of an N-palmitoyl moiety (headgroup fragments at m/z 378, 418, and 434) and two O-acyl groups (carboxyl anions at m/z 255 for 16:0 and m/z 279 for 18:2). It was shown previously that under these negative ion-FAB-MS/MS conditions, FFA from the sn-2 position are preferentially lost from the [M-86]$^-$ ions (equivalent to the phosphatidic acid fragment) for diacylphosphatidylcholines (Huang et al., 1992; Gage et al., 1994). Similar observations have now been made for the lipid classes diacylphospha-
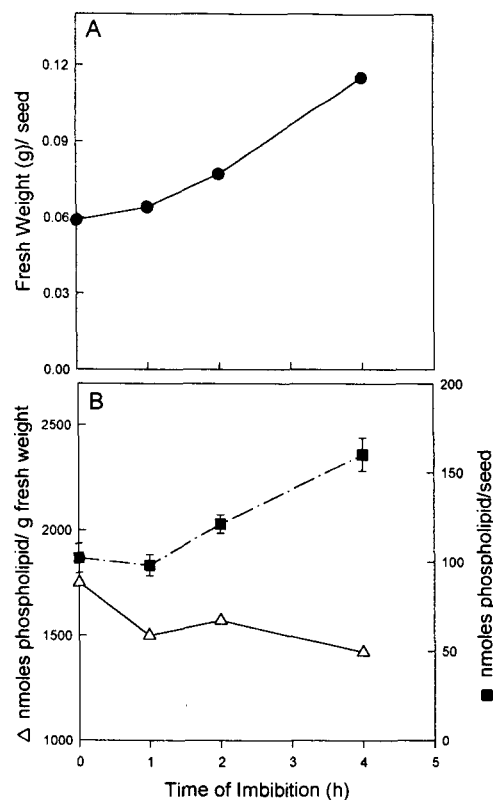
Figure 1. Time course of cottonseed imbibition. The uptake of water was measured by the increase in seed fresh weight with increasing time of imbibition (A). Total phospholipid content per gram fresh weight (△) and total phospholipid content per seed (■) are presented in B. Data points for A were from 30 seeds at each time point. Data points for B were means and SE from three separate experiments. SE values are shown only for the phospholipid/seed data.
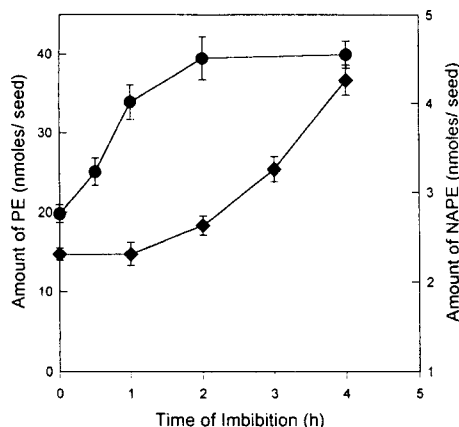
Figure 2. Time course of PE content (●) and NAPE content (♦) at several times during seed imbibition. Data points are means and SD of three separate experiments.

tidylglycerols, diacylphosphatidylinositols, diacylphos-phatidylserines, and N-acetyl diacylphosphatidylethano-lamines (Z.-H. Huang and D.A. Gage, unpublished data). Therefore, the preferential elimination of 18:2 as the FFA from the [M − H]⁻ ion, represented by m/z 672 (952 − 280) and 690 (952 − [280 − 18]), over that of 16:0 (represented by less intense peaks at m/z 696 (952 − 256) and 714 (952 − [256 − 18]) suggested that the 18:2 radyl is likely situated at the sn-2 position and the 16:0 O-acyl moiety is at the sn-1 position. Hence, the predicted structure for the molecular species m/z 952 [M − H]⁻ is N-palmitoyl 16:0/18:2-PE.

Based on MS/MS analyses of isolated cottonseed PE and NAPE (not shown, but similar to that in Fig. 3C), the main molecular species in the cottonseed mixtures have been assigned as summarized in Table I. The most abundant molecular ([M − H]⁻) ion of PE was at m/z 714 (represent-ing 16:0/18:2-PE) in both dry and imbibing cottonseeds. This also was the major "PE constituent" of NAPE molec-ular species [e.g. 16:0/18:2-PE(N-palmitoyl), 16:0/18:2-PE(N-linoleoyl)] in the dry and imbibing seeds. Palmitic and linoleic acids were consistently the chief N-acyl con-stituents of cottonseed NAPE. There was evidence that 19:0 was an O-acyl constituent of a relatively minor molecular species of NAPE at m/z 994, i.e. a fragment ion at m/z 297 was evident in the MS/MS spectrum. Although a molecu-lar species of PE also was identified with the same O-acyl configuration ([M − H]⁻ at m/z 732, 16:0/19:0-PE, data not shown), we have not confirmed the presence of this 19:0 by other analytical techniques. This observation is intriguing because cottonseed lipids are known to contain small quan-tities of cyclopropanoid fatty acids (van de Loo et al., 1993).

The specific activity of NAPE synthase was measured in cell extracts of seeds at different times after soaking (Fig. 5) and increased in a linear fashion with increasing time of imbibition from 35 pmol h⁻¹ mg⁻¹ protein in dry seeds to 129 pmol h⁻¹ mg⁻¹ protein in 4-h soaked seeds (approxi-mately a 4-fold increase). Based on an average NAPE syn-thase activity during imbibition of 82 pmol h⁻¹ mg⁻¹ pro-tein (which represented approximately 497 pmol h⁻¹ seed⁻¹; approximately 6.06 mg of protein was extracted per

seed), the NAPE synthase activity measured in homoge-nates in vitro is sufficient to account for the amount of accumulated NAPE in vivo (497 pmol h⁻¹ seed⁻¹ × 4 h = 1988 pmol NAPE/seed; this is very close to the actual increase of 1950 pmol of NAPE/seed quantified above, 4.26 nmol − 2.31 nmol). Because the extraction of lipids (and recovery of NAPE) from seeds (chloroform extraction of frozen, powdered seeds) is probably more complete rela-tive to the extraction of NAPE synthase enzyme activity (measured in filtered homogenates), the actual capacity for
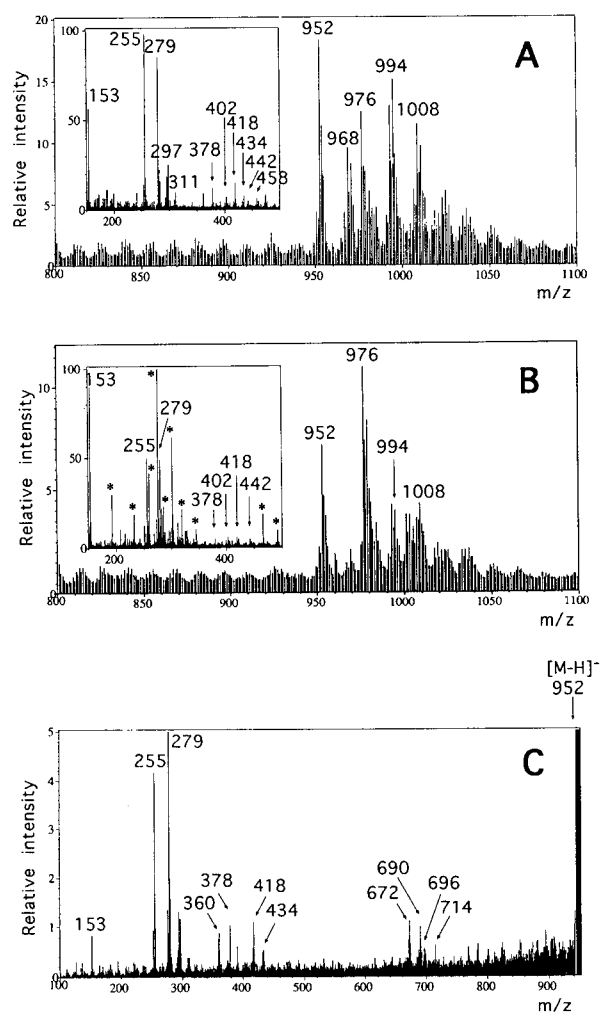

Figure 3. Negative-ion FAB-MS spectra of NAPE purified from dry (A) and 4-h-soaked (B) cottonseeds. Peaks due to the matrix, PEHA, are found at m/z 231, 259, 275, 285, 301, 318, 328, and 344 (designated by asterisks). Insets show the low-mass region of the spectra with headgroup (see Fig. 4) and acyl diagnostic fragments. The headgroup diagnostic ions are less pronounced in B due to the low concentration of the analyte in the sample. C, FAB-CAD-MS/MS spectrum of the [M − H]⁻ ion at m/z 952 in the dry seed. Diagnostic fragment ions at m/z 378, 418, and 438 (see Fig. 4) provided un-equivocal identification of the parent ion as an NAPE molecular species and revealed the N-acyl composition of this species. Fatty acid anion peaks at m/z 255 and 279 correspond to the O-acyl substituents 16:0 and 18:2, respectively.
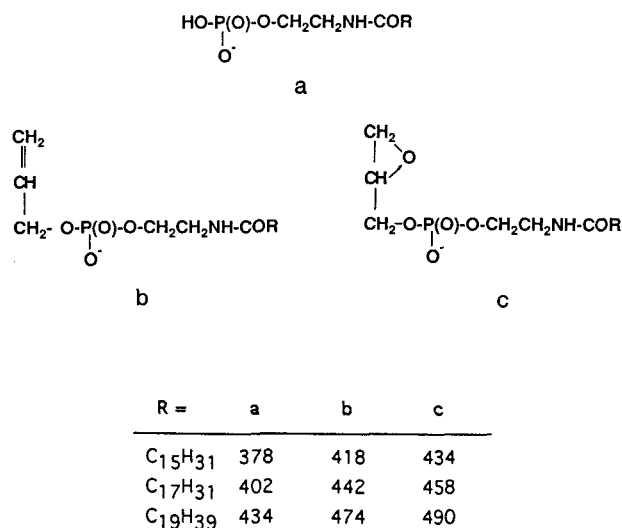
HO-P(O)-O-CH$_2$CH$_2$NH-COR

O$^-$

a

CH$_2$
‖
CH
|
CH$_2$- O-P(O)-O-CH$_2$CH$_2$NH-COR
O$^-$

b

CH$_2$
| \ O
CH /
|
CH$_2$-O-P(O)-O-CH$_2$CH$_2$NH-COR
O$^-$

c

R =	a	b	c
C$_{15}$H$_{31}$	378	418	434
C$_{17}$H$_{31}$	402	442	458
C$_{19}$H$_{39}$	434	474	490

Figure 4. Diagram of the deduced structures of [M − H]$^-$ diagnostic ions in FAB mass spectra of cottonseed NAPE. Structures represent the N-acylated derivatives of known diagnostic fragment ions derived from the PE headgroup.

NAPE biosynthesis in cottonseeds likely far exceeds the amounts of NAPE accumulated during seed imbibition.

DISCUSSION

It is becoming increasingly apparent that a variety of environmental stresses are manifested at the molecular level by damage to intracellular membranes due to the accumulation of FFA (including dehydration/rehydration, temperature stress, etc.; McKersie et al., 1988; Crowe et al., 1989a, 1989b; McKersie, 1991; Crowe et al., 1993; van Bilsen et al., 1994). The causal relationship is uncertain between the accumulation of FFA in membranes and the accumulation of highly reactive free radicals; however, both clearly promote the production of the other and both have damaging effects on the organism (McKersie, 1991; Liljenberg, 1992; LePrince et al., 1994). Recently, loss of seed (and pollen) viability has been correlated with the accumulation

Table I. *Selected molecular species of PE and NAPE in dry and imbibing cottonseeds identified by NI-FAB-MS/MS*

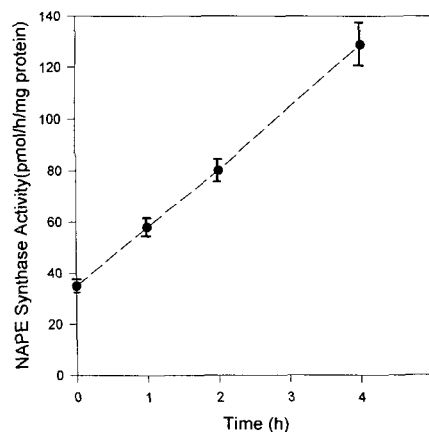| Type	Dry		Imbibing	
	[M-H]$^-$	Molecular species	[M-H]$^-$	Molecular species
PE	714[a]	16:0 /18:2-PE	714[a]	16:0 /18:2-PE
	738	18:2 /18:2-PE	738	18:2 /18:2-PE
NAPE	952[a]	N-palmitoyl 16:0 /18:2-PE	952[a]	N-palmitoyl 16:0 /18:2-PE
	976	N-linoleoyl 16:0 /18:2-PE N-palmitoyl 18:2 /18:2-PE	976[a]	N-linoleoyl 16:0 /18:2-PE N-palmitoyl 18:2 /18:2-PE
	994	N-palmitoyl 19:0 /18:2-PE N-linoleoyl 16:0 /19:0-PE		

[a] Indicates major species.

Figure 5. Time course of NAPE synthase specific activity at various times during seed imbibition. Activity was measured in seed homogenates (approximately 10 mg of protein) by following the specific incorporation of [1-^{14}C]palmitic acid into [^{14}C]NAPE. Assays were conducted in a final volume of 0.5 mL with shaking for 30 min at 45°C and stopped by the addition of boiling propanol. Lipids were extracted in chloroform, separated by TLC, and quantified by radiometric scanning (see "Materials and Methods").

of FFA (Crowe et al., 1989a; van Bilsen et al., 1994, and refs. therein). In fact, it has been proposed that the progression of senescence (normal developmental stage of plants) is a result of the accumulation of FFA in intracellular membranes and subsequent compromise of intracellular compartmentation (causing a cascade of other events including production of free radicals), eventually leading to cell death (McKersie, 1991). A concept appears to be emerging that a variety of seemingly different situations challenge plants at the cellular level in a similar manner. If this holds true, then the ability (or inability) of plants to respond to stress may be, in part, linked to their inherent ability to prevent accumulation of FFA in their membranes.

NAPE accumulates in intracellular membranes of damaged animal tissues (Schmid et al., 1990). Biophysical studies of aqueous dispersions of NAPE were interpreted to indicate that this lipid helps to stabilize a bilayer conformation under conditions in which nonlamellar phases would otherwise be favored (LaFrance et al., 1990; Schmid et al., 1990; Domingo et al., 1993). Together, these results suggest that NAPE plays a protective role in membranes and is synthesized in injured tissues of animals to help maintain intracellular membrane integrity (Schmid et al., 1990). By contrast, we discovered that NAPE was synthesized in vivo from [2-^{14}C]ethanolamine in plants under normal (nonstressed) physiological growth conditions (postgerminative growth of cotton seedlings) (Chapman and Moore, 1993a). It is interesting that when NAPE biosynthesis was reconstituted in vitro in microsomes of cotton cotyledons, PE was directly acylated with FFA to form NAPE (Chapman and Moore, 1993b, 1994; Chapman et al., 1995b). The synthesis of NAPE by this mechanism would produce a membrane phospholipid with bilayer-stabilizing properties, remove cytotoxic FFA from membranes, and reduce the concentration of PE (itself exhibiting a tendency

toward nonlamellar phases). It is possible that in cotton-seeds, membrane-bound NAPE synthase is a FFA scavenger involved in the protection of intracellular membranes from potential damage by FFA.

It is intriguing to speculate that NAPE synthase and NAPE play a general protective role in membranes of plants. Recent studies with tobacco cell suspensions support this notion (Chapman et al., 1995a); elicitation of documented defense responses by a fungal xylanase also resulted in a transient increase (approximately 3-fold) in NAPE content and NAPE synthase specific activity. NAPE molecular species are indeed present (Fig. 3; Table I) in dry seeds and accumulate during imbibition, a natural rehydration process that subjects plant cell membranes to significant osmotic forces. NAPE content increased during imbibition (Fig. 2) as did total phospholipid content (Fig. 1), such that the relative (perhaps vital) percentage of NAPE in dry seeds was similar to that in imbibing seeds (2.3 mol% compared to 2.6 mol%). NAPE synthase specific activity in seed extracts increased concomitantly with the increase in cottonseed NAPE content during seed imbibition (Fig. 5). The evidence gathered thus far supports a role for NAPE in the protection of intracellular membranes of plants, as has been suggested for animals; however, this evidence is circumstantial, and further work will be necessary to understand the precise function of NAPE synthesis in plants.

The synthesis of NAPE could further stabilize intracellular membranes by reducing the concentration of particular types of PE. PE with unsaturated acyl chains are not especially good bilayer-forming lipids. Rather, they tend to spontaneously form H_{II} phases at physiological temperatures (Yeagle, 1993). Steponkus and co-workers recently demonstrated that lethal damage of plant cells exposed to freezing temperatures was promoted by increased formation of H_{II}-phase lesions in plasma membranes and subsequent compromise of the membrane bilayer integrity (Uemura and Steponkus, 1994; Webb et al., 1994). Others have shown that N-acylation of PE raised the lamellar-to-H_{II}-phase transition temperature in mixed lipid systems (Schmid et al., 1990). The major molecular species of NAPE identified in imbibing cottonseeds was the molecular ion at m/z 976 (Fig. 3B; Table I). Although this species was present in dry seeds, its relative concentration (relative to other NAPEs) appeared to be increased most dramatically in the imbibing seeds, suggesting that the synthesis of this, or a few, molecular species of NAPE may be favored during cottonseed imbibition. MS/MS analyses indicated that the molecular ion at m/z 976 was a mixture of two molecular species of NAPE with the same overall fatty acid composition but a different positional distribution of these fatty acids [i.e. 16:0/18:2 PE (N-linoleoyl) and 18:2/18:2 PE (N-palmitoyl); Table I]. It is possible that the selective N-acylation of unsaturated PE species helps to stabilize the membrane bilayer of cells during seed dehydration/rehydration processes.

Mass spectrometric analytical procedures developed here provide a powerful tool for the unequivocal identification of PE and NAPE molecular species in plant lipid extracts. Triethanolamine has been used as the matrix of choice for the negative ion FAB analysis of phospholipids (Gage et al., 1994). However, it was noted that intense adduct ion peaks $[M - H + 26]^-$ and $[M - H + 42]^-$ were invariably formed in spectra of PE and other classes of phospholipids that contain a terminal NH_2, such as phosphatidylserine, ceramide aminoethylphosphonate, and sphingoethanolamine (Jensen and Gross, 1988; Matsubara and Hayashi, 1991). This can be misleading in the analyses of unknown lipid mixtures or in cases where the N-acetyl analogs may be present. The undesirable adducts were recently discovered (Z.-H. Huang and D.A. Gage, unpublished data) to be Schiff bases formed by the matrix-analyte interaction in the high-energy atom beams employed for ionization. To circumvent this shortcoming, we chose to use PEHA or PEHA-15-crown-5 alternative matrices. PEHA does not react with PE or other classes of amino-containing lipids under FAB conditions and is capable of producing high-quality mass spectra. Hence, we were able to obtain unambiguous molecular species information for both PE and NAPE in dry and imbibing cottonseeds. The new structural information provided here should facilitate more refined studies on the function, biosynthetic precursors, and enzymatic synthesis of this unusual phospholipid in membranes of plant cells.

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