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# Triacontanol and Jasmonic Acid Differentially Modulate the Lipid Organization as Evidenced by the Fluorescent Probe Behavior and $^{31}$P Nuclear Magnetic Resonance Shifts in Model Membranes

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**Abstract** Fluorescence resonance energy transfer (FRET), time-resolved fluorescence and anisotropy decays were determined in large unilamellar vesicles (LUVs) of egg phosphatidylcholine with the FRET pair *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine as donor and lissamine rhodamine B 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine as acceptor, using 2-ps pulses from a Ti:sapphire laser on LUVs with incorporated plant growth regulators: triacontanol (TRIA) and jasmonic acid (JA). FRET efficiency, energy transfer rate, rotation correlation time, microviscosity, and diffusion coefficient of lateral diffusion of lipids were calculated from these results. It was observed that TRIA and JA differentially modulated all parameters studied. The effect of JA in such modulations was always partially reversed by TRIA. Also, the generalized polarization of laurdan fluorescence indicated that JA enhances the degree of hydration in lipid bilayers to a larger extent than does TRIA. Solid-state $^{31}$P magic-angle spinning nuclear magnetic resonance spectra of LUVs showed two chemical shifts, at 0.009 and −11.988 ppm, at low temperatures (20°C), while at increasing temperatures (20–60°C) only one (at −11.988 ppm) was prominent and the other (0.009 ppm) gradually became obscure. However, LUVs with TRIA exhibited only one of the shifts at 0.353 ppm even at lower temperatures and JA did not affect the chemical shifts.

**Keywords** Fluorescence resonance energy transfer · Jasmonic acid · Membrane fluidity · $^{31}$P nuclear magnetic resonance · Time-resolved fluorescence · Time-resolved anisotropy · Triacontanol

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Triacontanol (TRIA), a saturated long-chain aliphatic alcohol ($C_{30}H_{61}OH$) (Fig. 1) is a natural component of plant epicuticular waxes (Ries et al. 1977). It has been known to be a potent plant growth promoting substance when sprayed, leading to increased growth and yield of many important agricultural and horticultural crops (Ries and Wert 1982; Ries and Houtz 1983; Muthuchelian et al. 1990; Srivastava and Sharma 1990; Misra and Srivastava 1991; Coumarane et al. 2000). A marked increase in the dry weight and level of reducing sugars, amino acids, soluble proteins and total nitrogen was observed in plants subjected to exogenous application of nanomolar doses of triacontanol (TRIA) (Ries 1985). Earlier studies have shown that TRIA increases the level of glycolipids in cotton (Shripathi and Swamy 1994) and alters the fluidity of membranes in vitro (Ivanov and Angelov 1997; Shripathi et al. 1997). TRIA also mediates the activation of some membrane-bound enzymes (Lesniak et al. 1986, 1989; Morré et al. 1991; Savithiry et al 1992). Further, experiments conducted in our laboratory, have shown that TRIA inhibits lipid peroxidation (Ramanarayan et al. 2000),

partially reverses jasmonic acid (JA)-stimulated proteinase inhibitors (Ramanarayan and Swamy 2004), and reverses the effect of JA on chlorophyll *a* fluorescence (Ramanarayan 2004). JA (Fig. 1) and its derivatives, described as jasmonates, are cyclopentanone derivatives that are ubiquitous in plants. Jasmonates are involved in regulation of plant growth and development such as in promotion of senescence, abscission, fruit ripening, and tendril coiling. JA has pervasive roles in seed germination, pollen development, and responses to mechanical and insect wounding, pathogen infection, abiotic stresses (Wastenack and Parthier 1997; Swamy 2005), and chlorophyll *a* fluorescence (Ramanarayan 2004). Further, some JA-induced responses are also modulated by other plant growth regulators (Sano et al. 1996; Tamogami et al. 1997; Rakwal and Komatsu 2001; Larher et al. 2002; Naik et al. 2002). The mechanism of action of these two growth regulators is not well understood. Interestingly, long-chain alcohols including TRIA are also known to exert a profound effect on animals such as regulating lipid metabolism in rats (Kato et al. 1995) and controlling inflammatory responses in animals (Warren et al. 1992; Carbajal et al. 1998).

In the present investigation, the dynamic changes in the lipid environment in model membranes with incorporated plant growth regulators TRIA and JA were studied by fluorescence resonance energy transfer (FRET), laurdan fluorescence, and solid-state $^{31}$P magic-angle spinning (MAS) nuclear magnetic resonance (NMR) measurements. Fluorescence spectroscopy provides useful information on the physical properties of lipids in membranes. Because of their great sensitivity and versatility, fluorescent lipid probes have been extensively used in the study of membranes (Lakowicz 1999; Maier et al. 2002). FRET involves the transfer of energy from one fluorophore (donor) to another fluorophore (acceptor) by a dipole-dipole interaction (Förster 1948). The FRET pair *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (N-NBD-PE; donor) and lissamine rhodamine B 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (L-RH-PE; acceptor) is used in the present study.

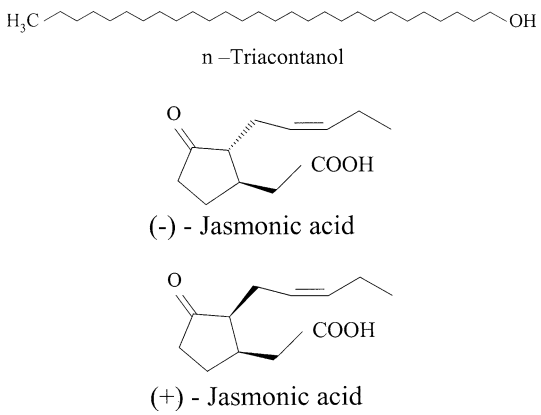


**Fig. 1** Chemical structures of triacontanol and chiral isomers of jasmonic acid

The fluorescence excitation and emission spectra of laurdan (6-lauroyl-2-dimethylaminonaphthalene) are very sensitive to polarity, reflecting the amount of water molecules found close to the probe and the dipolar dynamics (corresponding to how mobile these water molecules are) of its environment (Bagatolli et al. 1999; Parasassi et al. 1986). The interaction and molecular mechanisms of action of the plant growth regulators TRIA and JA, which differentially partition the hydrophobic core and the hydrophilic/aqueous environment, respectively, are defined by the changes in dipolar relaxation brought about by TRIA and JA and are measured by laurdan fluorescence.

To gain insight into the mechanism of action of TRIA and JA at the membrane level, we have investigated the conformational changes in the phospholipid bilayer brought about by the interaction of TRIA and JA with respect to changes in phosphocholine head mobility using solid-state $^{31}$P MAS NMR.

From earlier studies, the molecular mode of action and initial site of action for abscisic acid (ABA), gibberellins, and JA remain a matter of conjecture (Katzer and Stillwell 2003; Leshem et al. 1994; Finkelstein et al. 2002; Olszewski et al. 2002; Turner et al. 2002). The primary site of action is undisputedly the plasma membrane itself and no membrane-bound receptor has been found for JA until now (Turner et al. 2002), similarly to ABA (Finkelstein et al. 2002). In the present study, we employed biophysical techniques such as FRET, laurdan fluorescence, and solid-state $^{31}$P MAS NMR to demonstrate the potential of TRIA and JA to interact with membranes and to understand their association with membrane lipids. As the receptor protein has not been identified for some of these hormones even in the postgenomic era, it is essential to probe the lipid association of these growth regulators to elucidate their mechanism of actions in controlling plant growth and development. The recent observation that JA is involved in the suppression of cell proliferation in cancer cell lines and the recommendation of this compound as a potential anticancer drug (Balbi and Devoto 2008) add another dimension to the importance of studying the interaction of this compound with membranes.

## Materials and Methods

Egg phosphatidylcholine (PC) for the preparation of large unilamellar vesicles (LUVs) was extracted from egg yolk after precipitation with acetone, purified on Sephadex G-25, and further purified on silicic acid (Christie 1982).

NBD-PE and L-RH-PE were purchased from Molecular Probes (Eugene, OR, USA). Pure TRIA was a gift from BDK Co. (Hubli, India). JA [(±)-12,2$\beta$-3-oxo-2-(cis-2-pentyl) cyclopentane acetic acid] was purchased from Sigma (St. Louis, MO, USA). All other chemical reagents used were of analytical grade.

### Preparation of Large Unilamellar Vesicles

Liposomes were prepared from purified egg PC following the ether infusion method (Deamer and Bangham 1976). A known amount (1 mg/ml) of purified egg lecithin in chloroform along with appropriate amounts of FRET probes NBD-PE (1 mol%; donor) and L-RH-PE (0.25 mol%; acceptor) for FRET assay was cosolubilized with TRIA (equivalent to 2 mol% lipids), JA (0.1 mol% lipids), and a combination of TRIA and JA (2 mol% + 0.1 mol% lipids, respectively). The optimal concentration of TRIA and JA used was based on the maximum fluorescence intensity of the donor NBD-PE subjected to treatment with various amounts of TRIA and JA in egg PC liposomes. The sample was mixed well and dried under a stream of nitrogen/argon gas to form a thin film. The resultant thin film containing the membrane-forming lipid with FRET probes, TRIA and JA, was taken in a small volume of diethyl ether. After its complete solubilization the solution was injected into 10 ml of 0.01 M Tris-HCl (pH 7.0) buffer maintained at 55–65°C. The solvent was completely removed from the suspension under reduced pressure at 40°C. The resultant liposomes were LUVs (Deamer and Bangham 1976) and were used immediately after preparation.

### Fluorescence Measurements

Steady-state fluorescence measurements at temperatures from 293 to 323 K were carried out with a fluorescence spectrophotometer (Model F2000; Hitachi, Japan) with temperature controller accessories. The temperature of the assay samples in 1-cm-path-length quartz cuvette was controlled to ±1°C with a water-circulating bath. Steady-state FRET measurements were conducted by exciting NBD-PE (donor) at 465 nm and by recording the emissions of the donor and acceptor (L-RH-PE) at 530 and 575 nm, respectively. Absorption spectra were recorded using a UV/Vis spectrophotometer (Model 150-20; Hitachi, Japan).

### Time-Resolved FRET

Time-resolved fluorescence lifetime and anisotropy were measured using a time-correlated single-photon counting spectrometer comprised of a diode-pumped Nd-$YVO_4$ laser of 532 nm that was used as the excitation source for a titanium-sapphire mode-locked laser (Spectra Physics, USA). This provides tunable wavelengths from 720 to 1000 nm operating at a repetition rate of 4 MHz and a pulse width of <2 ps. The fundamental wavelength (750 nm) of the Ti:sapphire laser was used. The harmonic generator gives the second harmonic 375- to 435-nm output, which was used for excitation of the samples. The donor (NBD-PE) was excited at 425 nm and the fluorescence of the donor at 530 nm and that of the acceptor (L-RH-PE) at 575 nm was detected at the magic angle (54.7°) relative to the vertically polarized excitation beam by a high-gain Microchannel plate photomultiplier tube (Hamamatsu MCP-PMT R3890U). Fluorescence decay curves were obtained by collecting 10,000 counts in the peak channel. The time resolution in the collection of the fluorescence decay was 50 ps/channel for all time-resolved FRET measurements at temperatures from 293 to 323 K.

The fluorescence kinetic parameters (lifetime, amplitude, etc.) were obtained by deconvoluting the excitation instrument response function from the measured fluorescence decay. Data analysis was carried out using the software provided by the IBH (DAS-6), which was based on the reconvolution technique using he iterative nonlinear least-squares fit method. The goodness of fit between the observed decay and the calculated decay was judged by the reduced chi-square and weighted residual distribution. The fluorescence decay $I_C(t)$ is expressed as a sum of discrete exponentials (Lakowicz 1999):

$$I_C(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (1)$$

where $I$ is the number of discrete exponentials required to fit the decay profile, $\alpha_i$ and $\tau_i$ are the amplitudes and lifetimes, respectively, and the amplitudes represent the functional population of the fluorophores.

For measurement of time-resolved anisotropy the fluorescent intensity decays were collected with the emission polarizer kept at parallel (‖) and perpendicular (⊥) orientations with respect to the excitation polarizer. The polarized components were then corrected for G-factor, where G represents an instrument- and wavelength-dependent correction factor for the polarization bias of the detection system.

$$r(t) = \sum r_0 \exp\left(\frac{-t}{\phi}\right) \quad (2)$$

where $r_0$ is the initial anisotropy, which decays with the rotational correlation time $\phi$.

### Laurdan Generalized Polarization Measurements

The fluorescent probe laurdan, along with or without TRIA (2 mol%) and JA (0.1 mol%), was added to the chloroform

solution of lipid before thin film formation. The final concentration was 1:3000 (laurdan:lipid [PC]) = 0.4 mM. LUVs containing all the constituents were prepared by the ether infusion method as described earlier. Laurdan fluorescence spectra were acquired using an Hitachi F2000 fluorescence spectrophotometer with temperature control setup. Emission spectra were recorded at between 425 and 525 nm, with excitation at wavelengths of 340 and 410 nm, respectively.

Emission generalized polarization (GP) was calculated using

$$\text{Emission GP} = (I_{410} - I_{340})/(I_{410} + I_{340}) \quad (3)$$

where $I_{410}$ and $I_{340}$ are the emission intensities at 470 nm measured using excitation wavelengths of 410 and 340 nm, respectively (Parasassi et al. 1993).

### $^{31}$P NMR Measurements

LUVs were prepared by the method described earlier. The resultant liposomes (PC, 10 mg/ml) were subjected to sonication at 40 kHz for 10 min in a bath sonicator (D-7824 singen/htw; Elma, Germany) at 35°C. This uniformly dispersed liposome suspension was used for $^{31}$P NMR measurements.

Solid-state $^{31}$P MAS NMR spectra were recorded with a Bruker DSX300 Avance NMR spectrometer. The $^{31}$P resonance frequency was 121.44 MHz at a field strength of 7.05 T. The sample was carefully loaded into a $ZrO_2$ rotor in a MAS probe spinning at 5 kHz. The 90° pulse width was 5 μs, and high-power $^1$H decoupling was employed during acquisition of spectra. Between 1200 and 1500 scans were acquired, with an interpulse delay of 5 s and a line broadening value of 100 Hz. The sample temperature was regulated by flowing heated air, which was controlled by the MAS-probe heat assembly. A 10-min equilibration delay was allowed between experiments at different temperatures. Chemical shift assignments were externally referenced relative to 85% orthophosphoric acid at 0.00 ppm.

## Results

### Time-Resolved Fluorescence Resonance Energy Transfer

Changes in the bilayer environment brought about by TRIA and JA, either individually or in combination (TRIA + JA), were studied by the excited-state lifetimes of the donor N-NBD-PE and the acceptor N-LRH-PE. From Table 1, it is evident that the donor lifetime in the absence and presence of acceptor was biexponential. An overall decrease in the lifetime of the donor probe N-NBD-PE in the presence of the acceptor N-LRH-PE was observed, which decreased further with an increase in temperature. LUVs with TRIA showed a decreased excited-state lifetime of donor and acceptor. An increase in the lifetime of FRET probes was observed in PC vesicles with JA. Further, in vesicles with incorporated TRIA + JA, an increase in the lifetime decay rate or a decrease in the excited-state lifetime of the donor N-NBD-PE and acceptor N-LRH-PE was observed compared to those in vesicles containing JA. These variations in excited-state lifetimes indicate a possible role of these plant growth regulators in modulating membrane fluidity.

Further, the time-resolved energy transfer efficiency was calculated. For this, the average lifetimes of the donor N-NBD-PE in the absence and presence of the acceptor N-LRH-PE was used. The average lifetimes (data not shown) were calculated using the expression

$$\tau_{AV} = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2} \quad (4)$$

where $\alpha_1$ and $\alpha_2$ are the pre-exponential factors associated with the two lifetime components, $\tau_1$ and $\tau_2$ of the donor in the absence and presence of the acceptor. The time-resolved energy transfer efficiency ($E$) was calculated from the time-dependent donor decay in the absence of the acceptor ($\tau_D$) and presence of the acceptor ($\tau_{DA}$):

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \quad (5)$$

An increase in the energy transfer efficiency was observed in LUVs with incorporated TRIA (Fig. 2). The transfer efficiency decreased in vesicles with incorporated JA compared to LUVs with TRIA and with TRIA + JA (Fig. 2). Further, vesicles with TRIA + JA exhibited an increased transfer efficiency, more or less equivalent to that of LUVs containing TRIA. The time-resolved transfer efficiencies are dependent on the rate of donor decay. TRIA and TRIA + JA contribute to the increase in the rate of donor decay (decrease in the excited-state lifetime) and thereby result in increased transfer efficiency (Fig. 2).

The rate of energy transfer ($k_t$) in LUVs with incorporated TRIA, JA, and TRIA + JA was also calculated, using

$$(k_t) = \frac{1}{\tau_D}\int\left(\frac{r}{R_0}\right)^6 \alpha_a 2\pi\, rdr = \frac{\pi\,\sigma_A R_0^6}{2\,r^4} \quad (6)$$

where $\sigma_A$ is the concentration of the acceptor, $r$ is the distance of closest approach, $\tau_D$ is the donor decay time, and $R_0$ is the Förster distance. An increase in the energy transfer rate was observed in liposomes with TRIA, JA, and TRIA + JA at between 20 and 35°C; it was found to decrease linearly at higher temperatures, from 35 to 50°C. Since the energy transfer rate depends on the overlap integral and also the donor decay time, the differences in energy transfer rate observed appear to be due to a decrease

**Table 1** Fluorescence lifetimes (nanoseconds) of donor and acceptor, independently, in LUVs containing JA, TRIA, and TRIA + JA at different temperatures: $\tau_1$ and $\tau_2$ are the fluorescence lifetime components of biexponential decay

| | Lifetime at | | | |
|---|---|---|---|---|
| | 20°C | 30°C | 40°C | 50°C |
| *Control* | | | | |
| Donor | | | | |
| $\tau_1$ | 1.21 ± 0.0243 | 1.04 ± 0.0224 | 0.89 ± 0.0255 | 0.60 ± 0.0171 |
| $\tau_2$ | 4.31 ± 0.0168 | 3.59 ± 0.0180 | 3.32 ± 0.0175 | 2.66 ± 0.0135 |
| $\chi^2$ | 1.2106 | 1.1246 | 1.2115 | 1.3130 |
| Acceptor | | | | |
| $\tau_1$ | 0.54 ± 0.0238 | 0.41 ± 0.0087 | 0.38 ± 0.0195 | 0.36 ± 0.010 |
| $\tau_2$ | 3.58 ± 0.0062 | 3.09 ± 0.0053 | 2.70 ± 0.0056 | 2.37 ± 0.0056 |
| $\chi^2$ | 1.3534 | 1.2929 | 1.3441 | 1.4954 |
| *JA* | | | | |
| Donor | | | | |
| $\tau_1$ | 1.39 ± 0.0267 | 1.12 ± 0.0288 | 0.95 ± 0.0263 | 0.54 ± 0.0165 |
| $\tau_2$ | 4.62 ± 0.0180 | 3.85 ± 0.0171 | 3.33 ± 0.0150 | 2.65 ± 0.0125 |
| $\chi^2$ | 1.1754 | 1.1246 | 1.2631 | 1.3904 |
| Acceptor | | | | |
| $\tau_1$ | 0.64 ± 0.0236 | 0.48 ± 0.0214 | 0.40 ± 0.0216 | 0.35 ± 0.0198 |
| $\tau_2$ | 3.75 ± 0.00607 | 3.27 ± 0.0061 | 2.65 ± 0.0055 | 2.01 ± 0.00553 |
| $\chi^2$ | 1.1637 | 1.4634 | 1.3239 | 1.2920 |
| *TRIA* | | | | |
| Donor | | | | |
| $\tau_1$ | 1.12 ± 0.0264 | 0.91 ± 0.0191 | 0.87 ± 0.0205 | 0.50 ± 0.0189 |
| $\tau_2$ | 4.16 ± 0.0174 | 3.55 ± 0.0155 | 3.19 ± 0.0150 | 2.35 ± 0.0163 |
| $\chi^2$ | 1.2913 | 1.1246 | 1.0979 | 1.3550 |
| Acceptor | | | | |
| $\tau_1$ | 0.46 ± 0.0177 | 0.38 ± 0.0216 | 0.33 ± 0.0192 | 0.21 ± 0.0157 |
| $\tau_2$ | 3.43 ± 0.0056 | 3.00 ± 0.00561 | 2.49 ± 0.0055 | 1.92 ± 0.0045 |
| $\chi^2$ | 1.3078 | 1.3337 | 1.4010 | 1.4954 |
| *TRIA + JA* | | | | |
| Donor | | | | |
| $\tau_1$ | 1.29 ± 0.0255 | 1.02 ± 0.0244 | 0.90 ± 0.024 | 0.49 ± 0.017 |
| $\tau_2$ | 4.29 ± 0.0161 | 3.68 ± 0.0171 | 3.20 ± 0.0147 | 2.49 ± 0.0155 |
| $\chi^2$ | 1.0998 | 1.1753 | 1.2621 | 1.234 |
| Acceptor | | | | |
| $\tau_1$ | 0.57 ± 0.021 | 0.47 ± 0.020 | 0.35 ± 0.0187 | 0.24 ± 0.020 |
| $\tau_2$ | 3.62 ± 0.006 | 3.10 ± 0.0060 | 2.53 ± 0.0052 | 1.90 ± 0.005 |
| $\chi^2$ | 1.2265 | 1.3624 | 1.2888 | 1.3920 |

*Note*: *LUVs* large unilamellar vesicles, *JA* jasmonic acid, *TRIA* triacontanol

in the Förster distance and also the lifetimes of the donor (Table 1) in LUVs with TRIA, JA, and TRIA + JA at higher temperatures (35–50°C). Electrostatic interactions will also alter the distances between the donor and the acceptor, which may lead to a change in the transfer rate and efficiency (Lakowicz 1999, Thomas et al. 1978).

## Time-Resolved Anisotropy Decay Measurements

To get more information on the molecular order and dynamics of the membrane, the time-resolved anisotropies of the donor N-NBD-PE and the acceptor N-LRH-PE in the absence or presence of TRIA, JA, or TRIA + JA in LUVs were measured at temperatures of from 20 to 50°C. The anisotropy decay of the donor and the acceptor was best represented by a single-exponential decay with a single rotational correlation time ($\phi$) (Table 2). The microviscosity of the system under study and the temperature had a direct influence on the rotational correlation time ($\phi$) (Table 2). A decrease in $\phi$ was correlated with a decrease in the microviscosity of the PC LUVs, which decreased further with increases in temperature.

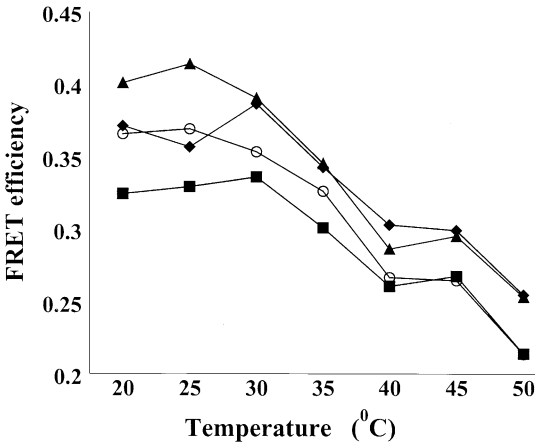


**Fig. 2** Time-resolved FRET efficiency measurements in LUVs without additions (*open circle*) and LUVs containing TRIA (*filled triangle*), JA (*filled square*), and TRIA + JA (*filled diamond*) at different temperatures

The results presented in Table 2 indicate the changes in microviscosity and $\phi$ of LUVs with incorporated TRIA, JA, or TRIA + JA. A decrease in microviscosity with a decrease in $\phi$ was observed in vesicles containing TRIA. In LUVs with JA, an increase in microviscosity was correlated with an increase in $\phi$. Further, a shorter $\phi$ was also observed in vesicles with TRIA + JA compared to vesicles with incorporated JA (Table 2). The alterations in microviscosity and variations in $\phi$ of LUVs caused by these plant growth regulators strongly suggest their differential intercalation in model membranes, which in turn is supported by their respective chemical structures.

In general, the Förster distance is affected by the diffusive motions of energy transfer probes in the membrane. These diffusive motions further depend on the state of the membrane. Rapid diffusions among the probes may affect the energy transfer rate and thereby the energy transfer efficiency. Hence, the diffusion coefficient ($D$) was calculated (Cantor and Schimmel 1980) considering the microviscosity of the LUVs with incorporated plant growth regulators,

$$D = kT/6V_{\mathrm{h}}\eta \tag{7}$$

The diffusion coefficient was observed to increase in vesicles with JA and decrease in vesicles with TRIA. On the

**Table 2** Fluorescence parameters and physical properties of lipid bilayers derived from time-resolved anisotropy and lifetime measured at different temperatures in LUVs containing TRIA, JA, and TRIA + JA

| | Control | JA | TRIA | TRIA + JA |
|---|---|---|---|---|
| Energy transfer rate, photons $s^{-1} \times 10^{10}$ | | | | |
| 20°C | 1.72 | 2.47 | 1.76 | 2.89 |
| 25°C | 1.99 | 2.8 | 2.02 | 3.15 |
| 30°C | 2.4 | 3.69 | 3.25 | 4.17 |
| 35°C | 2.36 | 3.56 | 2.86 | 3.63 |
| 40°C | 2.25 | 3.45 | 2.77 | 3.55 |
| 45°C | 2.11 | 3.38 | 2.61 | 3.40 |
| 50°C | 3.01 | 3.13 | 2.50 | 3.31 |
| Rotation correlation time of donor in presence of acceptor ($\phi$), ns | | | | |
| 20°C | 0.93 ± 0.109 | 1.27 ± 0.019 | 0.86 ± 0.119 | 1.19 ± 0.090 |
| $\chi^2$ | 1.082 | 1.2457 | 1.1646 | 0.8047 |
| 30°C | 0.89 ± 0.082 | 1.19 ± 0.11 | 0.76 ± 0.0125 | 1.13 ± 0.0511 |
| $\chi^2$ | 1.2026 | 1.208 | 0.1768 | 0.8555 |
| 40°C | 0.80 ± 0.078 | 1.09 ± 0.135 | 0.73 ± 0.058 | 0.93 ± 0.071 |
| $\chi^2$ | 1.0903 | 1.1588 | 1.1631 | 1.0311 |
| 50°C | 0.65 ± 0.044 | 0.85 ± 0.047 | 0.60 ± 0.053 | 0.80 ± 0.083 |
| $\chi^2$ | 1.2054 | 0.8157 | 1.1015 | 1.3436 |
| Microviscosity ($\eta$), cP | | | | |
| 20°C | 1.84 | 2.51 | 1.70 | 2.35 |
| 30°C | 1.70 | 2.28 | 1.45 | 2.16 |
| 40°C | 1.48 | 2.02 | 1.35 | 1.72 |
| 50°C | 1.17 | 1.53 | 1.08 | 1.44 |
| Diffusion coefficient ($D$), $cm^2\ s^{-1} \times 10^{-9}$ | | | | |
| 20°C | 3.05 | 3.30 | 2.24 | 2.39 |
| 30°C | 3.41 | 4.00 | 2.55 | 2.69 |
| 40°C | 4.05 | 4.44 | 2.97 | 3.49 |
| 50°C | 5.31 | 5.75 | 4.06 | 4.31 |

*Note*: *LUVs* large unilamellar vesicles, *JA* jasmonic acid, *TRIA* triacontanol. Goodness of fit is represented by the respective Chi-square values

other hand, the calculated values for $D$ also showed an increase in LUVs with TRIA + JA (Table 2). In all cases the values of $D$ increased with increases in temperature (Table 2).

It should be noted that the diffusive motions are dependent on the decay rates of the donor probes. Diffusion coefficients can only be calculated for donors with longer excited-state lifetimes in which extensive diffusion can occur (rapid diffusion limit, $D\tau_0/r^2 \gg 1$, where $D$ is the sum of the diffusion coefficients of the donor and the acceptor, $\tau_0$ is the lifetime of the donor in the absence of transfer, and $r$ is the mean distance between donor and acceptor). However, in the present case, fluorescence donors having excited-state lifetimes in the nanosecond range, characteristic of most energy transfer studies, were considered. This allows donor-to-acceptor diffusion, which was analyzed by a static limit ($D\tau_0/r^2 \gg 1$).

## Phosphorus Magic-Angle Spinning Nuclear Magnetic Resonance

High-speed MAS was studied under conditions similar to those used for fluorescence studies except that the lipid concentration was kept high (10 mg/ml) to resolve the phosphorus signals. Since the lipid head group mobility and the phase transition are temperature dependent, $^{31}$P MAS NMR spectra from PC liposomes were recorded over a range of temperatures from 20 to 60°C. The spectra showed two phosphorus resonances at 20°C, at 0.009 and −11.988 ppm (Fig. 3). The phosphorus resonance at 0.009 ppm gradually decreased with increases in temperature and became almost undetectable at 60°C (Fig. 3a–e), representing the probable decrease in the chemical shift anisotropy as a result of alterations in the membrane phase. However, when the temperature was reduced from 60 to 20°C, the spectral component, which was almost undetectable at 60°C, became prominent and a similar reduction of temperature in TRIA-containing liposomes did not bring about any change in the spectral component (results not shown). $^{31}$P MAS NMR spectra of liposomes with TRIA, JA, and TRIA + JA were recorded at temperatures of 40 and 50°C. With JA (Fig. 4b, d, e) there was no change in the two phosphorus resonances at either of these temperatures. However, in liposomes with TRIA (Fig. 4a, d, e) and TRIA + JA (Fig. 4c d, e), the phosphorus resonance at 0.353 ppm (TRIA) and 0.123 ppm (TRIA + JA) was prominent, and the other spectral component of phosphorus, at −11.988 ppm, did not appear at 40 and 50°C. Further, there was no change in the spectral behavior with a decrease in temperature in vesicles with incorporated TRIA and JA (results not shown). This indicates an averaging of chemical shift anisotropy of phosphorus and changes in membrane fluidity brought about by TRIA.

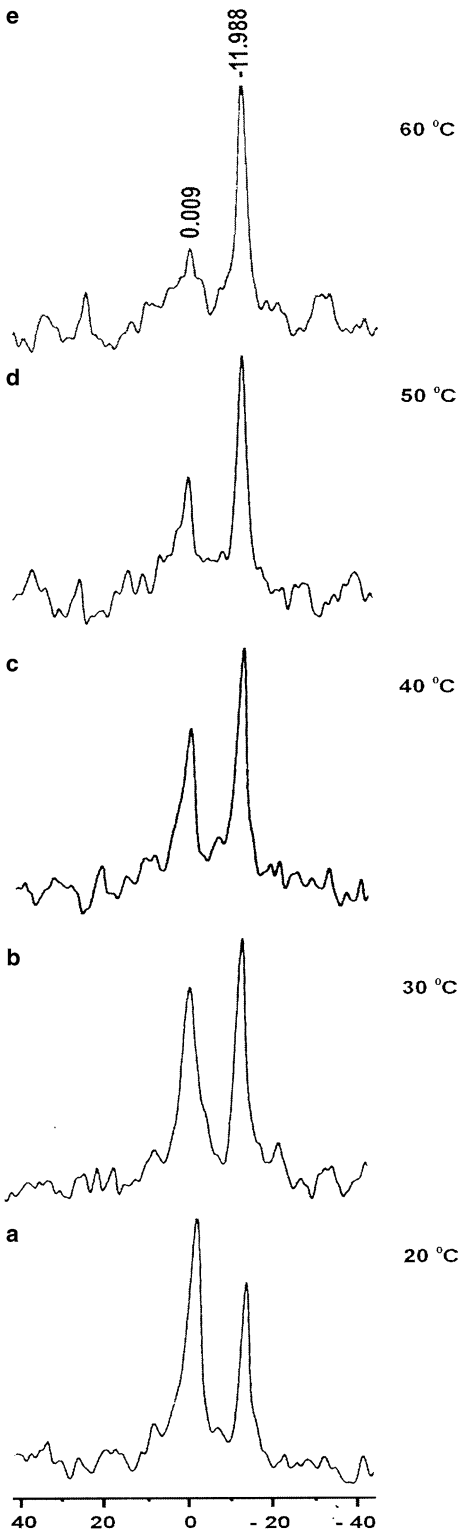


**Fig. 3** Solid-state $^{31}$P MAS NMR spectra of LUVs at different temperatures. The scale represents parts per million

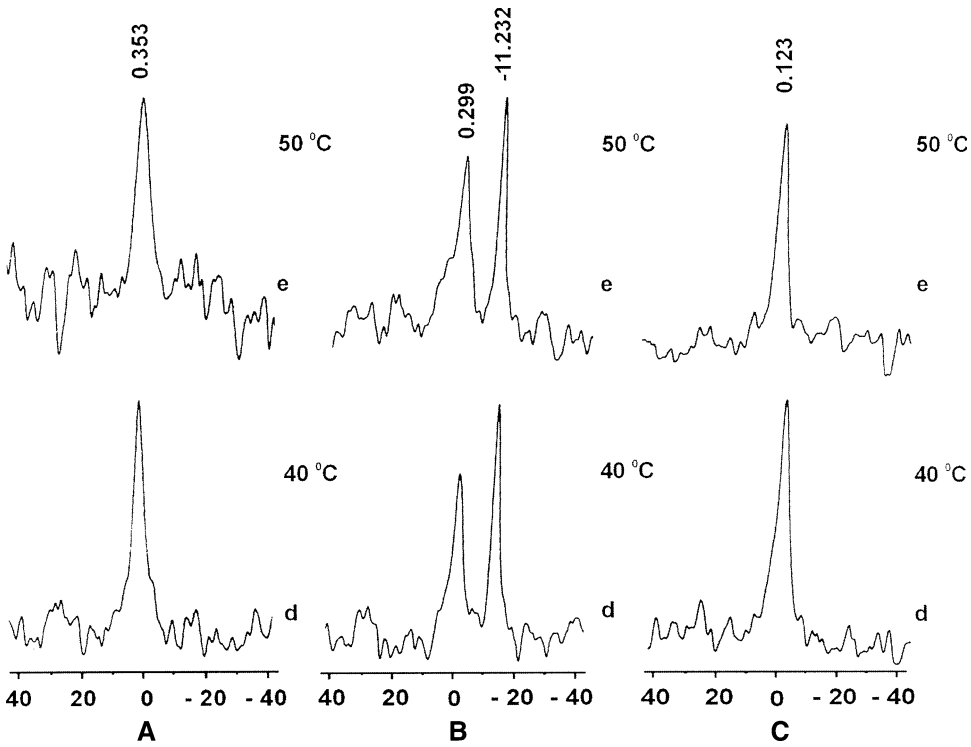


**Fig. 4** Solid-state $^{31}$P MAS NMR spectra of LUVs containing TRIA (**A**), JA (**B**), and TRIA + JA (**C**) at 40 and 50°C. The scale represents parts per million

### Effect of Triacontanol and Jasmonic Acid on Laurdan Generalized Polarization

The GP of laurdan was calculated from the spectral scans of liposomes containing TRIA, JA, and TRIA + JA at different temperatures ranging from 20 to 50°C. Figure 5 shows a decrease in the the emission GP (emGP) values in egg PC liposomes containing TRIA, JA, and TRIA + JA compared to the control. The effect of JA in decreasing the emGP was much higher compared to that of TRIA and TRIA + JA (Fig. 5). The decrease in emGP values indicates a concomitant increase in the degree of hydration of the bilayer. Further, JA was able to increase the hydration of the bilayer compared to TRIA and TRIA + JA (Fig. 5).

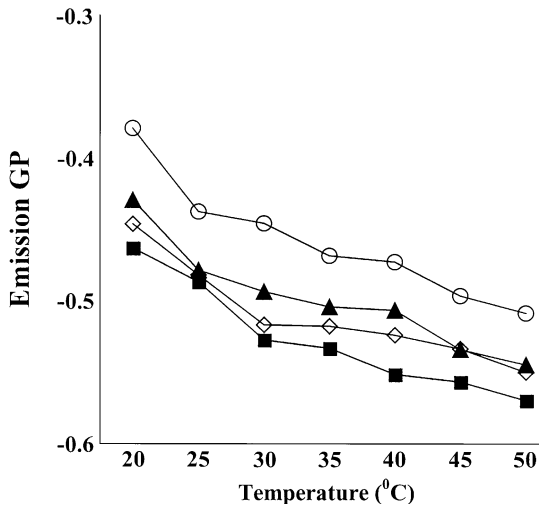


**Fig. 5** Emission generalized polarization of laurdan in LUVs without additions (*open circle*) and LUVs with TRIA (*filled triangle*), JA (*filled square*), and TRIA + JA (*open diamond*) at different temperatures

## Discussion

TRIA has been shown to be a potent growth promoter (Ries and Houtz 1983), whereas JA acts as a senescing agent in plants (Wasternack and Parthier 1997; Swamy 2005). Apart from the varied biochemical evidence on the regulation of physiological responses by TRIA and JA (Ries et al. 1977; Ries and Wert 1982; Ries et al. 1990; Muthuchelian et al. 1990; Srivastava and Sharma 1990; Misra and Srivastava 1991; Ries 1985; Shripathi and Swamy 1994; Lesniak et al. 1986, 1989; Morré et al. 1991; Savithiry et al. 1992; Swamy 2005; Koda 1992), the information available on their interaction and the molecular mechanism of their action is rather limited. From the biophysical point of view, earlier reports on natural and artificial membranes have demonstrated that the structural properties of these membranes may be strongly affected by the presence of membrane-associated molecules such as drugs and anesthetics (Lee 1976, 2003) and, also, plant growth regulators (Shripathi et al. 1997; Pauls et al. 1982; Burner et al. 1993; Leshem et al. 1994; Videira et al. 1999; Katzer and Stillwell 2003). Moreover, an increased rate of

energy transfer and transfer efficiency is observed if donor and acceptor partition into the same membrane phase, and vice versa, relative to that expected for a random distribution of donors and acceptors (Lakowicz 1999). The lipid bilayer is an anisotropic medium, where lipid movements are much slower than in a nonviscous medium and largely constrained to two dimensions, which also influences energy transfer efficiency between donors and acceptors. From Table 1, it is clear that the time-resolved lifetime of the donor in the absence and presence of the acceptor is biexponential. This decay complexity is, however, common for fluorescent probes in interactions with microheterogeneous systems (Loura et al. 1996). A decrease in the fluorescence lifetimes of both the donor and the acceptor caused by TRIA, and also as a function of increasing temperature, was observed (Table 1). It is well documented that fluorophores exhibit longer average lifetimes in a low-temperature gel-phase lipid environment and shorter average lifetimes in the fluid liquid crystalline phase (Shripathi et al. 1997; Lentz et al. 1976; Lentz 1993; Ruggiero and Hudson 1989). Further, TRIA might act as a modulating agent by its intercalation in the membrane interior and bring about changes in the lipid phase around the fluorophores, resulting in a decrease in the lifetimes of the donor and acceptor probes (Table 1). These results were further confirmed by time-resolved anisotropy measurements, where TRIA decreased the microviscosity of the lipid bilayer, thereby decreasing the $\varphi$ of the donor and acceptor probes (Table 2). This decrease in microviscosity and $\phi$ caused by TRIA also correlates with the increased diffusion coefficient ($D$), which might also contribute to the efficient rate of energy transfer, resulting in improved energy transfer efficiency (Table 2). Furthermore, because TRIA is a 30-carbon-chain compound and would be considered a membrane-spanning molecule, its fluidizing or disordering effect could also be explained in terms of a large diffusion coefficient as reported for molecules which span the hydrophobic core of the bilayer (Nadler et al. 1985). These TRIA-induced fluidity changes can also be attributed to the stimulation of membrane-bound enzymes (Lesniak et al. 1986, 1989; Savithiry et al. 1992), thus stimulating rapid physiological responses.

The amount of information available on the regulation of plant growth and development by JA is profoundly increasing. However, its initial site of action in plants, which triggers a cascade of metabolic events, has remained unresolved until now. Thus the phase changes induced by JA in PC membranes with incorporated FRET probes can serve as a sensitive indicator of the extent to which JA can alter the bilayer and thereby regulate cellular processes. It should be noted here that JA, being partially hydrophobic (Fig. 1), is precluded from deep penetration into the bilayer. Hence the greatest effect appears to be close to the surface of the bilayer. The increased $\varphi$ correlated with a decrease in $D$ (Table 2) further ensures comparatively less disordering of the bilayer by JA. From these observations it is clear that JA results in membrane perturbance which is of a different magnitude than the fluidizing effect of TRIA. Earlier results on other plant hormones such as indole acetic acid, gibberellins, and ABA, and also the drought-induced phenolic compound arbutin, have shown different perturbing effects caused by these substances, even though they have preferential binding in the hydrophilic/hydrophobic interphase (Pauls et al. 1982; Stillwell and Wassall 1993; Hincha et al. 1999). The partitioning of these growth hormones and arbutin would alter the membrane lipid phase and thereby influence membrane properties such as permeability and microheterogeneity that regulate the cellular processes. This is in agreement with our present results on JA-induced perturbations by partitioning in the polar region of the phospholipid bilayer (Table 2). Earlier results of Parasassi et al. (1990a, b) have shown the alteration of membrane heterogeneity by ABA. The exact reversal of the TRIA-induced changes by JA, or vice versa, observed here might be due to changes in membrane heterogeneity brought about by the modulation of the physical state of membrane lipids by TRIA and JA (Tables 1, 2). It may be noted that various membrane-incorporated molecular compounds such as cholesterol, peptides, insecticides, and drugs are able to alter the physical membrane structure and functions by the formation of dynamic lipid domains at various length and time scales (Begelson et al. 1995; Mouritsen and Jørgensen 1995). Based on this, possible domain formation can be expected for TRIA, which is also an anti-inflammatory (Kato et al. 1995; Warren et al. 1992; Carbajal et al. 1998; Ramanarayan et al. 2000) molecule in addition to being a potent plant growth regulator. Hence the interaction of TRIA and JA by lipid domain formation could also affect the partitioning of donors and acceptors, resulting in variations in energy transfer efficiencies.

The preferential partitioning of TRIA and JA was further confirmed by our results on LUVs with incorporated TRIA + JA. Earlier reports indicated an altered membrane microheterogeneity caused by ABA (Parasassi et al. 1990a, b), an alteration in the basic membrane properties caused by plant sterols (Demel and de Kruijff 1976), and an increase in the indole acetic acid-induced permeability (Misso et al. 1986) and cryoprotective nature of arbutin in thylakoid membranes due to alterations in the physical state of membrane lipids induced by arbutin (Hincha et al. 1999). Based on this wide array of experiments and on our results, it may be proposed that the physiological responses triggered by TRIA and JA are linked to their differential association with lipid components of the membrane.

Laurdan is a membrane probe that displays spectral sensitivity to the polarity of the environment (Parasassi

et al. 1990a, b). Upon excitation, the dipole moment of the laurdan molecule increases substantially and water molecules localized near the probe, at the level of the phospholipid glycerol backbone, will rotate to adapt to this new dipole, thereby lowering the excitation energy state of laurdan, which is termed dipolar relaxation (Parasassi et al. 1990a, b). The rate of dipolar relaxation depends on the freedom of rotation of water molecules (Parasassi and Gratton 1995). Thus emission spectra of laurdan mainly provide information on the mobility of water molecules in the membrane interphase region, while excitation spectra elucidate the amount of water in the membrane interphase (Parasassi et al. 1990a, b). This unique property of laurdan has been used to detect drug-induced fluid phase domains in model membranes (Balasubramanian et al. 2002), bilayer-to-hexagonal phase transitions (Epand and Leon 1992), effects of cationic lipids on liposome physical properties (Campbell et al. 2001), changes in bilayer microheterogeneity caused by ABA, and cochleate phase formation as a result of interaction of cations with negatively charged lipids like phosphatidylserine (Ramani and Balasubramanian 2003). To quantify the behavior of laurdan fluorescence and thus the relaxation of water molecules in the membrane, the concept of GP was used (Parasassi et al. 1990a, b). Single-point emGP was calculated from the emission spectra at temperatures of from 20 to 50°C (Fig. 5). The increased molecular mobility of water molecules at the hydrophilic/hydrophobic interphase resulting in the increased dipolar relaxation and higher degree of hydration of the membrane might be due to the existence of an alternative conformation of the polar groups, which could form a hydrophilic, channel-like surface normal to the bilayer plane that would allow facile penetration of water molecules along the length of JA, thereby exposing the adjacent lipid surfaces to increased interaction with water, similar to that suggested for ABA (Katzer and Stillwell 2003). This is quite expected due to its selective positioning in the membrane interphase and its interaction with the glycerol backbone. The decrease in the excitation GP and emGP observed in LUVs with TRIA and TRIA + JA might be due to a reduced molecular mobility of water molecules. In vesicles with TRIA + JA, the increase in molecular mobility induced by JA is partially reversed by TRIA (Fig. 5). Further, the state of lipids in the membrane at different temperatures also contributes to dipolar relaxation (Parasassi et al. 1995), which was observed as a decrease in the emGP at temperatures ranging from 20 to 50°C (Fig. 5). Hence, a higher GP value indicates a more rigid membrane with a low rate of molecular mobility, and vice versa. Unlike ABA, which decreases the molecular mobility of bilayer polar residues and increases microheterogeneity (Parasassi et al. 1990a, b), JA appears to increase the molecular mobility and also alter the microheterogeneity of the system.

Earlier studies on phosphate head group mobility using various NMR methods have shown the lipid polar groups to act as a "molecular electrometer" responding not only to molecules that partition into the lipid bilayer but also to any process that modifies the electrical properties of membranes (Seelig et al. 1987). It has also been shown that polyhydroxy compounds like trehalose, sorbitol, and glycerol have little effect on head group structure (Seelig et al. 1987). Further, the interaction of trichloroethylene, an inhalational general anesthetic, with PC model membranes (Forrest and Rodham 1985), $\beta$-purothionine intercalation with DMPG vesicles (Richard et al. 2003), mastoparan-induced changes in phospholipid membranes (Hori et al. 1999), and phospholipid-cholesterol interactions have been extensively studied by $^{31}$P NMR and solid-state $^{31}$P MAS NMR. It is well known that other plant growth regulators, such as indole acetic acid, ABA, and gibberellins, physically interact with membrane lipids (Shripathi et al. 1997; Pauls et al. 1982; Forrest and Rodham 1985; Parasassi et al. 1990a, b; Stillwell et al. 1990; Stilwell and Wassall 1993; Katzer and Stillwell 2003). Based on the various physiological processes exercised by TRIA and JA and on the present findings, it would be reasonable to correlate their mechanism of action with their differential effect on the bilayer. In light of these investigations, $^{31}$P MAS NMR was used to examine the phosphate mobility in LUVs with incorporated TRIA, JA, and TRIA + JA (Figs. 3, 4). The lipid bilayer undergoes phase transition involving changes in one or several types of order, such as lipid-molecule translational order and rippling (Mouritsen and Jørgensen 1994). From the present results (Fig. 3a–e), it is quite evident that one of the two phosphorus resonances at 0.009 ppm decreased gradually and became almost undetectable with an increase in temperature from 20 to 50°C, corresponding to the changes in phosphorus head group mobility. The two observed spectral components (0.009 and −11.988 ppm) (Fig. 3a–d) of phosphorus might be due to two magnetically inequivalent orientations of PC. This, however, correlates with similar earlier results of Seelig (1978) pertaining to phosphatidylethanolamine in unit cells. Similarly, this unique behavior of the spectral components of phosphorus in 18:0/18:0 PC liposomes was also observed by Cullis et al. (1976). Further, at lower temperatures, all the motions that could average the $^{31}$P shielding tensor or chemical shift anisotropy are slow (Fuldner 1981), thus resulting in this unique occurrence of two spectral components of phosphorus in LUVs subjected to $^{31}$P MAS NMR measurements (Fig. 3). At higher temperatures or temperatures corresponding to phase transition, the rate of reorientation of the head group may change due to rapid axial rotation of the phosphocholine residue

(Cullis and de Kruijff 1976), resulting in a decrease in chemical shift anisotropy and thus contributing to the single spectral component of phosphorus (Fig. 3e).

It must be noted here that earlier studies have shown that TRIA was able to decrease the phase transition temperature and alter the fluidity in protoplast membranes due to its lipophilic nature (Ivanov and Angelov 1997; Shripathi et al. 1997). An increase in fluidity caused by TRIA renders the hydrocarbon core to be more disordered, and this might increase the axial rotation of the phosphocholine head group, resulting in decreased chemical shift anisotropy and the corresponding single spectral component of phosphorus (Fig. 4a, d, e). On the contrary, JA, being partially hydrophobic and having its pentyl group partially aligned near the polar head region, might reduce the motional freedom of the polar head in the hydrophilic/hydrophobic interphase. This might result in an alteration of chemical shift anisotropy exhibiting two spectral components (0.299 and −11.232 ppm) (Fig. 4b, d, e), very similar to the phosphorus resonance at lower temperatures (Fig. 3). TRIA + JA would further reduce the chemical shift anisotropy, which appears to be due to the increase in axial rotation of the polar head groups, resulting in a single spectral component (0.123 ppm) of phosphorus. This indicates the differential ability of both plant growth regulators to alter membrane fluidity. The present results seem to offer a satisfactory explanation for the pleiotropic biological effects brought about by TRIA and JA and, also, the reversal of JA-induced effects by TRIA, or vice versa.

Several investigations with both natural and phospholipid membrane systems have shown that the plant growth regulators auxin, gibberellin, ABA, and TRIA can affect membrane properties (Shripathi et al. 1997; Burner et al. 1993). The involvement of membrane-bound receptor proteins mediating the action of ABA, gibberellins, TRIA, and JA has not been identified even in this postgenomic era (Shripathi et al. 1997; Finkelstein et al. 2002; Olszewski et al. 2002; Turner et al. 2002). Earlier studies from this laboratory on the interaction of TRIA and JA at the biochemical level show that porcine pancreas trypsin and gut proteinases of the insect *Spodoptera litura* were inhibited in the presence of leaf proteins extracted after treatment with JA, and spraying the leaf with TRIA reversed this proteinase inhibitor effect (Ramanarayan and Swamy 2004). Further, the present biophysical changes show a remarkable correlation with our earlier biochemical observations, wherein TRIA reversed the effect of JA in inducing proteinase inhibitors in tomato leaves, which was considered to be a result of interaction in the process of JA signaling (Ramanarayan and Swamy 2004). In addition, the suppression of the primary photochemical activity in sorghum (*Sorghum bicolor* L. Moench) leaves caused by JA treatment is reversed by TRIA, thus further establishing the antagonistic effects of these two growth regulators (Ramanarayan 2004). In light of these investigations it is reasonable to suggest that the mechanisms of action of both TRIA and JA, at least in part, involve lipid mediation, and these growth regulators do not necessarily act directly through amembrane-bound receptor protein to elicit various physiological responses as has been suggested for ABA (Finkelstein et al. 2002). JA has been shown to induce mitochondrial membrane depolarization and release of cytochrome *c* from the mitochondria of cancer cells leading to cell death (Balbi and Devoto 2008). Further, JA is also able to produce reactive oxygen species in cancer cells (Balbi and Devoto 2008), and on the other hand, TRIA has been shown to suppress lipid peroxidation (Ramanarayan et al. 2000). TRIA's reversal of the effects of JA on membrane lipid organization as evidenced by fluorescence probe behavior and $^{31}$P NMR described in the present paper appears to reflect the functional behavior of these two compounds.

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