Structural Consequences of Genetically Engineered Saturation of the Fatty Acids of Phosphatidylglycerol in Tobacco Thylakoid Membranes. An FTIR Study†

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ABSTRACT: The role of phosphatidylglycerol (PG) in protein−lipid interactions and membrane dynamics has been studied in the thylakoids of wild type and manipulated tobacco plants transformed with complementary DNAs for glycerol-3-phosphate acyltransferases (GPATs) from squash and *Arabidopsis*. The expression of the foreign enzymes resulted in the level of saturation of the PG molecules being higher in the squash and lower in the *Arabidopsis* transformants, as compared with the level in wild-type tobacco. For the analysis of fatty acyl chain dynamics in the thylakoid membranes, the $\nu_{sym}CH_2$ vibration bands of the infrared specta were decomposed into two components, corresponding to ordered and disordered fatty acyl chain segments. With this approach, it was shown that in squash GPAT-transformed tobacco thylakoids a rigid lipid domain exists below 25 °C. Above 25 °C, the dynamics of all thylakoid membranes were very similar, regardless of the manipulations. PG seems to tune the dynamics at the protein−lipid interface rather than to affect the structure of the proteins directly. Above 50 °C, the frequencies of the disordered $\nu_{sym}CH_2$ component bands were decreased. This lipid-related phenomenon correlated with protein denaturing. It is demonstrated that the protein aggregation appearing upon heat denaturing changes the conformational distribution of the disordered lipid population. The data also reveal that the protein stability does not depend on the fatty acid composition of the PG molecules; other lipids should provide the environment governing the protein stability in the thylakoid membrane. This is the first such detailed analysis of the infrared spectra of biological membranes that permits a differentiation between structurally different lipid populations within a membrane.

Thylakoid membranes, delicate assemblies of proteins, pigments, and lipids, are able to transform the physical energy of light into chemical energy, and hence into organic compounds. Besides the specific proteins and the pigments, the lipid composition of the thylakoid membranes is also uniquely specific. This is regarded as being essential for the functioning of these membranes when they adapt to altering environmental conditions, e.g., to temperature stresses. The phase transition of membrane lipids and the chilling sensitivity of plants were long ago postulated to be related (*1*, *2*). It was thought that the membrane lipids of chilling-sensitive plants enter the gel phase at chilling temperatures, whereas those of chilling-tolerant plants remain in the liquid-crystalline phase. This hypothesis was tested in cyanobacteria by growing different species at different temperatures: it was found that the phase transition temperatures of their membranes and the temperatures critical for chilling-induced damage shifted in parallel with the growth temperatures (*3*).

In higher plants, the phase transition of membrane lipids such as that observed in cyanobacteria has not been seen. Here, a correlation between the chilling sensitivity and the extent of unsaturation of the fatty acids of thylakoid membrane lipids was found only when the individual lipid classes were analyzed. In the chloroplast membranes of herbaceous plants, there was a clear correlation between the chilling sensitivity and the level of saturated and *trans*-monounsaturated molecular species (known as "high-melting point" molecular species) of phosphatidylglycerol (PG)[1] (*4*, *5*). A similar correlation has been observed for a wide variety of higher plants (*5*−*10*). These molecular species undergo the gel-to-liquid-crystalline phase transition around room temperature, as demonstrated on isolated PG molecules in a model system (*11*). *In situ*, however, the phase transition of thylakoid membranes has not been detected in the physiological temperature range. This failure might have been due to the small amount (∼5−10% of the total) of high-melting point PG in the thylakoid membrane and by the imperfect specificity of the methods that were applied.

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[1] Abbreviations: ARAB, *Arabidopsis*; FTIR, Fourier transform infrared; GPAT, glycerol-3-phosphate acyltransferase; PG, phosphatidylglycerol; SQUA, squash; TOAR, tobacco plant transformed with *Arabidopsis* cDNA for glycerol-3-phosphate acyltransferase; TOBA, tobacco; TOSQ, tobacco plant transformed with squash cDNA for glycerol-3-phosphate acyltransferase.

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In the study presented here, we have modified the former attempts in two ways. (i) By means of genetic manipulations, we altered the fatty acid content of PG in tobacco, which allowed us to look for differences between wild-type and mutant tobacco thylakoids. This approach can be more sensitive than searching for a small, unknown phenomenon. (ii) We used Fourier transform infrared (FTIR) spectroscopy, a noninvasive and very sensitive technique, to analyze lipid disorder, protein secondary structure, protein−lipid interactions, and membrane dynamics.

With regard to the first of these modifications, glycerol-3-phosphate acyltransferase (GPAT) in chloroplasts catalyzes the transfer of the acyl group of acyl-bound acyl carrier protein (ACP) to the *sn*-1 position of glycerol 3-phosphate (*12*). In chilling-tolerant plants, this enzyme specifically uses 18:1-ACP as its substrate, while in chilling-sensitive plants, it uses both 18:1-ACP and 16:0-ACP (*13*, *14*). Tobacco was manipulated by transformation with complementary DNAs (cDNAs) for GPAT from squash and *Arabidopsis*, and the chilling sensitivities of the transformed tobacco plants changed in accordance with the character of squash and *Arabidopsis* (*15*).

With regard to the second of the modifications, FTIR spectroscopy has proven to be a valuable tool in the analysis of lipid order, protein secondary structure, and lipid−protein interactions. For the study of lipid order, mostly the frequency shift of the symmetric stretching vibration of the CH_2 groups ($\nu_{sym}CH_2$) has been utilized (*16*, *17*). We recently demonstrated, however, that the $\nu_{sym}CH_2$ frequency shift in response to increasing lipid disorder is a consequence of the change in relative intensities of two competing $\nu_{sym}CH_2$ component bands (*18*). These two components have been assigned to *trans* and *gauche* segments of the lipid fatty acyl chains. *Gauche* segments in membrane lipids may appear because of either protein−lipid interactions or temperature-induced membrane dynamics. These contributions could be separated by recording FTIR spectra as a function of temperature (*19*). By further exploiting this approach, we observed a carotenoid-rigidified lipid population in the thylakoids of a cyanobacterium (*20*).

With the help of cDNA for squash GPAT, we manipulated the PG molecules in tobacco in such a way that their saturated, *trans*-monounsaturated fatty acid content was increased considerably. With the same enzyme from *Arabidopsis*, more unsaturated PG molecules were incorporated into the tobacco thylakoid membranes. Then, we made use of FTIR spectroscopy to compare the lipid disorder, membrane dynamics, and lipid−protein interactions in the thylakoid membranes of the transformed and wild-type tobacco plants.

MATERIALS AND METHODS

Plant Material. Transgenic tobacco plants (*Nicotiana tabacum* var. Samsun) were obtained as described previously (*15*). All plants were grown according to the method of Moon et al. (*21*).

Sample Nomenclature. The following abbreviations will be used in this paper: ARAB, *Arabidopsis*; SQUA, squash; TOBA, tobacco; TOAR, tobacco transformed with *Arabidopsis* cDNA for glycerol-3-phosphate acyltransferase; and TOSQ, tobacco transformed with squash cDNA for glycerol-3-phosphate acyltransferase. These names will be used in all those situations relating to measurements within this work; in other contexts, the standard names of the plants will be used.

Thylakoid Preparation. Thylakoid membranes were prepared according to the method of Leegood and Malkin (*22*), with the modification that the thylakoid membranes obtained with their method were further purified on a stepwise gradient made with 21, 27, 45, and 60% Percoll (Pharmacia, Uppsala, Sweden) in a 50 mM Tris-HCl buffer (pH 7.8) containing 0.3 M sucrose, 20 mM NaCl, and 5 mM $MgCl_2$. The gradient was centrifuged at 13000*g* for 30 min. After centrifugation, the thylakoid membranes were collected from the Percoll gradient, resuspended in the same buffer, and collected by centrifugation. The thylakoid membranes were stored at −80 °C until the FTIR measurements were taken.

Analysis of Thylakoid Membrane Lipids. Lipids were extracted from the isolated thylakoid membranes according to Bligh and Dyer (*23*). Classes of polar lipids were separated by ion-exchange column chromatography on silica gel, and by thin-layer chromatography on silica gel (*4*). The separated lipids were esterified, and their methyl esters were analyzed by gas chromatography (*4*).

Infrared Measurements. For infrared measurements, 100 μL aliquots of thylakoid membrane suspensions were diluted with 1 mL of D_2O-base 10 mM HEPES buffer (pD 7.0), centrifuged for 15 min with a Beckman TL100 centrifuge at 85 000 rpm, resuspended in 1 mL of the same buffer, and centrifuged again. Thylakoids were placed between CaF_2 windows, separated by a 25 μm thick Teflon spacer. The windows were put into a sample holder, mounted on a shuttle device. The temperature of the sample holder could be set by a computer-controlled water bath. The accuracy of the temperature setting was better than 0.5 °C.

The temperature-dependent measurements were performed in the range of 5−60 °C by repeating the following measurement cycle: recording the background, recording the sample spectrum, measuring the actual temperature on the surface of the CaF_2 window, setting the new temperature (in 2−3 °C steps), and waiting for 7 min for the new thermal equilibrium to be established. The whole measurement cycle was computer-controlled. Infrared spectra were recorded on an IFS66 (BRUKER, Karlsruhe, Germany) FTIR spectrometer equipped with a liquid nitrogen-cooled MCT detector, at 2 cm^{-1} spectral resolution, in the interval of 900−4000 cm^{-1}; 2048 interferograms were averaged for each sample and each background spectrum.

Data processing and spectrum analysis were carried out by using SPSERV software (Cs. Bagyinka, Institute of Biophysics, Biological Research Center, Szeged, Hungary). A detailed description of the fitting procedure is given in ref *18*.

RESULTS

Changes in the Lipid and Fatty Acid Compositions of the Thylakoid Membranes of the Transgenic Plants. We previously transformed tobacco plants with a binary plasmid pSQ, in which a cDNA encoding the mature protein of squash GPAT was fused to a chloroplast-targeting signal and placed under the control of the cauliflower mosaic virus 35S promoter. This allowed the overexpression of the squash and

the *Arabidopsis* enzyme in the chloroplasts of transgenic plants (*15*). As a consequence, the fatty acid composition of the PG changed considerably, and in the expected direction: the *cis*-unsaturated fatty acid content of ~32 mol % in the TOBA decreased to 12 mol % in the TOSQ plants and increased slightly to 36 mol % in the TOAR plants. (The relative amounts of the *cis*-unsaturated fatty acids in squash and *Arabidopsis* were 18 and 40 mol %, respectively.) The relative amounts of the lipid classes were not affected by the genetic manipulation (*15*).

Structural Investigations on Thylakoid Membranes. The structures of the thylakoid membranes were studied by FTIR spectroscopy. The temperature was introduced as an additional parameter, whereby differences in the phase properties of the lipids, in the membrane dynamics, in the protein structure, etc., between the different plants could be explored. For the analysis of the structure and dynamics of the thylakoid membranes, two regions of the FTIR spectra were used: the C−H stretching region from 3050 to 2800 cm^{-1} and the region from 1800 to 1600 cm^{-1}, including the ester C═O and amide I vibrations.

Analysis of the C−H Stretching Region. The C−H stretching regions (Figure 1A) of the infrared spectra of the thylakoids, involving contributions mostly from the fatty acyl chains of the membrane lipids, were very similar in the three plants. Nevertheless, the $\nu_{sym}CH_2$ band for TOSQ is at a somewhat lower frequency, indicating more ordered fatty acyl chains here as compared with TOBA and TOAR.

To learn more about the organization of the thylakoid membranes and the role of lipids in it, a detailed analysis of the infrared spectra is needed. As we have shown previously (*18*), the $\nu_{sym}CH_2$ band can be fitted with two components, one corresponding to ordered and the other to disordered fatty acyl chain segments. We adopted that approach here, and fitted two components to the $\nu_{sym}CH_2$ bands in the infrared spectra. As an example (Figure 1B), the result of such a fit is shown for thylakoid membranes prepared from TOSQ. The extremely small residuals at ~2850 cm^{-1} should be noted (see the inset in Figure 1B). The two components involved in the analysis of the $\nu_{sym}CH_2$ band are drawn with thicker lines and labeled with the letters ***O*** and ***D***, standing for ordered and disordered, respectively.

For the fitting of a series of spectra recorded as a function of increasing temperature, the strategy was as follows. Since the ***O*** component may be expected to "melt" away with increasing temperatures, the parameters of the ***O*** component (frequency and width) were kept constant after their determination from spectra recorded at 5 °C. The ***O*** component was most intensive at this temperature, and thus, its parameters could be determined most accurately here. This approximation does not allow distribution over different conformations for the ***O*** component. In contrast, all parameters of the ***D*** component were left free since they represent fatty acyl chain segments that should necessarily have a conformational distribution, and this distribution may change with increasing temperatures. To permit the comparison of different experiments, each spectrum was normalized with the integral of its fitted region (e.g., 3050−2820 cm^{-1} for C−H stretching).

The results of such fits for the thylakoid membranes of the three plants are shown in Figure 2. (For comparison, the results obtained with conventional, one-component evaluation and the differences between the one- and two-component analyses are given as Supporting Information.) The ratios of the intensities (intensity = the integral of the component) of the ***O*** components to the ***D*** components are given in Figure 2A as a function of temperature. We chose this ratio because it permits a comparison of the different membranes. These ratios may shift, however, due to varying protein/lipid ratios of the membranes, since the protein CH_2 and CH_3 groups contribute to a lipid phase-insensitive background here. At a higher protein/lipid ratio in a membrane, higher values and a lower temperature sensitivity may be expected for the ***O***/***D*** ratios. In the present case, the protein/lipid ratios were fairly similar in the three plants (*vide infra*).

As may be seen in Figure 2A, TOBA exhibits only a very slight decrease in the ***O***/***D*** ratio between 5 and 25 °C, and the ratio then levels off. TOAR exhibits a lower ***O***/***D*** ratio with no breakpoint at all. At low temperatures, the relative amount of the ***O*** component is highest in TOSQ. In TOSQ,

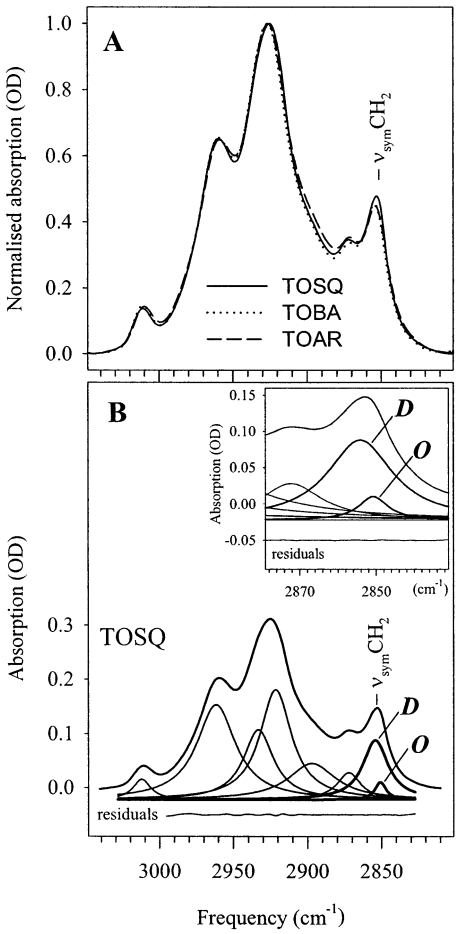

FIGURE 1: Analysis of the C−H stretching region of the infrared spectra of wild-type tobacco (TOBA) and tobacco transformed with the cDNA of squash GPAT (TOSQ) and of *Arabidopsis* GPAT (TOAR). (A) Comparison of the C−H regions of the thylakoid membranes of TOBA, TOSQ, and TOAR. The spectra as shown were subjected to linear baseline subtraction between 3050 and 2800 cm^{-1}, and to normalization to the amplitude of the band at ~2920 cm^{-1}. (B) Decomposition of the C−H region as demonstrated on thylakoid membranes of TOSQ. Component bands are depicted on the calculated constant background needed for the fit. The ***O*** (ordered) and ***D*** (disordered) components discussed in this paper in relation to membrane structure and dynamics are drawn with thicker lines. Spectra were recorded at 5 °C. Residuals are displaced for clarity. For details of the fitting procedure, see the text.

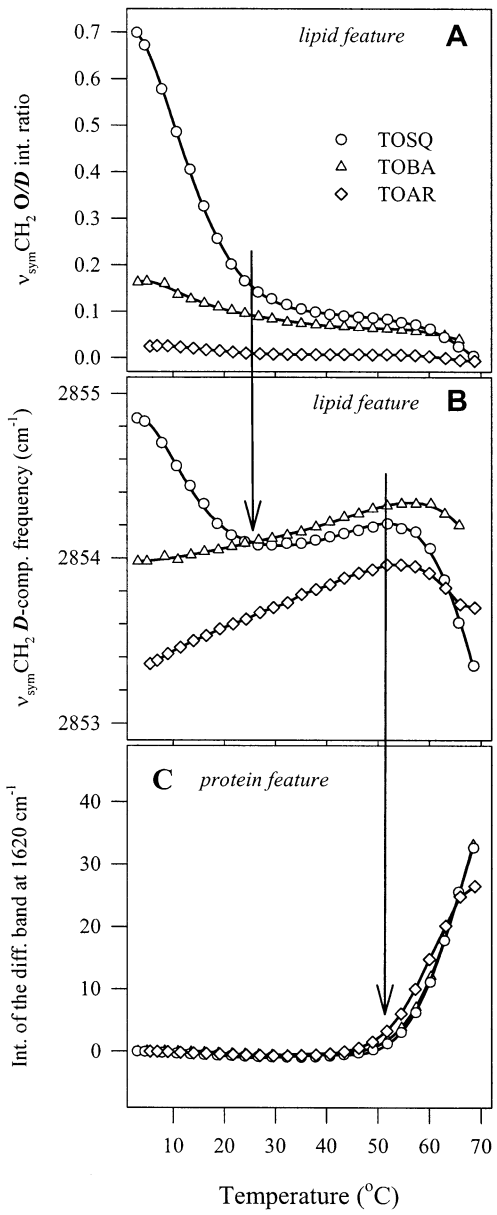

FIGURE 2: Correlation between lipid and protein dynamics in the thylakoid membranes of wild-type and genetically manipulated tobacco plants. (A) Temperature dependence of the intensity ratios of the ***O*** and ***D*** components of the $\nu_{sym}CH_2$ band, (B) frequencies of the ***D*** components, and (C) heat-induced denaturing of the membrane proteins as characterized by the increasing intensity of the difference band at ~1620 cm^{-1} (see Figure 4) assigned to intermolecular β-sheets formed upon protein aggregation.

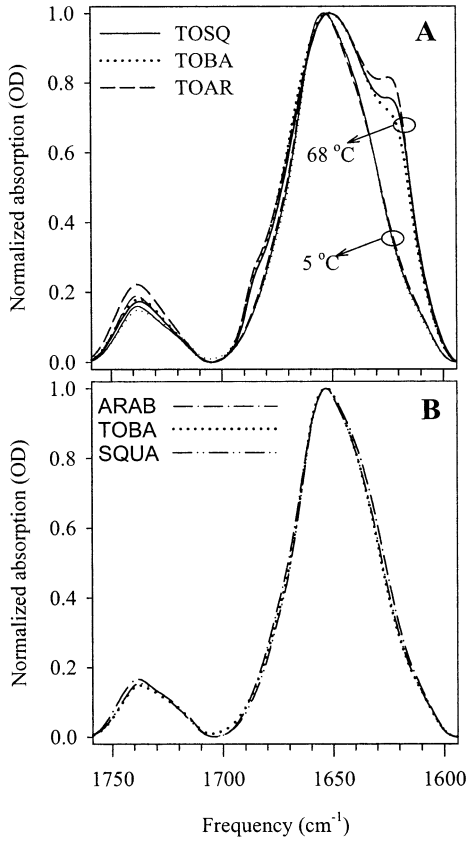

FIGURE 3: Amide I and ester C═O stretching region of the thylakoid membranes of the plants that were studied. (A) Comparison of the spectra of wild-type tobacco (TOBA), tobacco transformed with the cDNA of squash GPAT (TOSQ), and tobacco transformed with the cDNA of *Arabidopsis* GPAT (TOAR) at 5 and 68 °C. (B) Comparison of spectra of tobacco (TOBA), squash (SQUA), and *Arabidopsis* (ARAB) recorded at 5 °C. The spectra as shown were normalized after baseline subtraction; the baseline was a Gaussian curve fitted to the regions of 1770−1767, 1709−1706, 1597−1594, 1507−1505, and 1365−1362 cm^{-1}. The major section of the spectrum is not shown.

as shown above, the level of saturated fatty acyl chains in the PG molecules is considerably higher than in TOBA (76% of the PG molecules in TOSQ vs 36% in TOBA). The further slight decrease in the ***O***/***D*** ratios above 50 °C may be related to changes in the structure of the membrane proteins and will be discussed later.

There are considerable differences between the three plants studied in the temperature dependence of the frequency of the ***D*** component. This frequency is expected to shift upward with an increase in the level of temperature-induced lipid fatty acyl chain disorder at higher temperatures. Indeed, there is a tendency to shift toward higher values in the frequencies of the ***D*** components for all three membranes. It is noteworthy, however, that while the increase in the ***D*** frequency for TOAR starts immediately at 5 °C, for TOBA there is a slightly lower rate of increase up to ~25 °C and for TOSQ there is a considerable decrease before a slow increase starts. It is interesting that changes in the temperature courses of the ***D*** frequency curves in TOBA and TOSQ coincide with the completion of the "melting" of the ***O*** components (see the arrow between panels A and B of Figure 2).

Moreover, there is a breakdown in the ***D*** frequency in each of the three plants in the temperature region of 50−60 °C. If only the fatty acyl chain disorder is considered, such a phenomenon should mean increasing order of the chains in this temperature range. If only lipids were present in the investigated system, such a phenomenon would be impossible. Therefore, the behavior of other membrane components, i.e., proteins, should also be investigated, since their denaturing may be expected to occur in this temperature range, affecting the lipid phase.

Structure and Heat Denaturing of Thylakoid Membrane Proteins. Figure 3A depicts the 1760−1600 cm^{-1} region of the infrared spectra of the three tobacco-based plants. The amide I region (1700−1600 cm^{-1}) is characteristic of the secondary structure of proteins. Close to the amide I region, at ~1725−1740 cm^{-1}, are the bands of ester C═O vibrations. These bonds are present due to the binding of fatty acids to

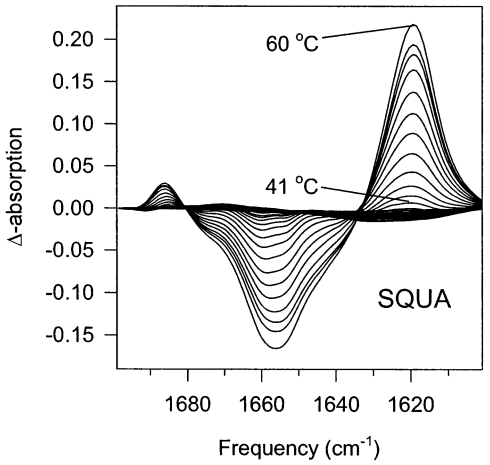

FIGURE 4: Heat-induced difference spectra in the amide I and ester C═O region, illustrated on squash thylakoid membranes. The differences were calculated from the spectra as shown in Figure 3A by subtracting the spectrum recorded at 5 °C from each spectrum registered at increasing temperatures. In the analysis of the protein stabilities, the intensity of the difference band at ~1620 cm^{-1} was taken as a measure of the extent of protein denaturing.

glycerol in the phospholipids and of phytol chains to the chlorophylls. Since there are no such ester C═O groups in proteins, the amide I to ester C═O band intensity ratio is a good measure of the changes in the relative amounts of the proteinic and lipidic components of the membranes. The strong similarity of the three spectra recorded at 5 °C indicates that, as intended, the genetic manipulation affected only the fatty acid composition of the membranes, the protein components and the protein/lipid ratio remaining unchanged.

The relative amounts of the lipids in the tobacco-based plants did not vary considerably either, as can be seen from the intensities of the ester C═O bands at ~1740 cm^{-1} (Figure 3A). (The somewhat higher-frequency ester C═O bands in the high-temperature spectra are misleading, since for better visual comparison of the changes in the amide I region the spectra were normalized at their maximal amplitudes, which does not take into account the increased width of the amide I bands in the spectra recorded at 68 °C. The estimation of the relative amount of proteins to lipids should be based on the ratio of the integrated intensities and not of the amplitudes of the amide I and ester C═O bands.)

Heat denaturing induces characteristic features in the amide I region of the infrared spectra of both water-soluble and membrane proteins. These involve the appearance of a strong band and a weaker band, at ~1620 and ~1684 cm^{-1}, respectively (*24*, *25*). The evolution of these bands was also seen at increasing temperatures, as shown in Figure 3A.

For a detailed characterization of the process of heat denaturing, the 1700−1600 cm^{-1} region of the thylakoid infrared spectrum was considered. Difference spectra were calculated by subtracting the first spectrum of a temperature run from those recorded at increasing temperatures. The evolution of the denaturing-related spectral changes in these difference spectra is shown for SQUA in Figure 4. There is a noteworthy parallel evolution of the 1620 and 1684 cm^{-1} bands, characteristic of intermolecular β-structures. The source of these structures is the degradation, mostly of α-helices, as shown by the deepening of a negative band at ~1658 cm^{-1} with increasing temperature.

To quantify the progress of heat denaturing, a Lorentzian curve was fitted to the 1620 cm^{-1} band emerging in the difference spectra. The intensities of these bands as a function of temperature for TOSQ, TOBA, and TOAR are plotted in Figure 2C. To allow a comparison of the different plants, before calculation of the difference spectra, each spectrum was normalized with its own intensity integrated between 1700 and 1594 cm^{-1}. Thus, the intensity of the 1620 cm^{-1} difference band can be directly related to the denatured fraction of the membrane proteins. Therefore, the thermotropic responses of the 1620 cm^{-1} band intensities can reveal the differences among the three plants with respect to the denaturing of their thylakoid membrane proteins. According to Figure 2C, the proteins in the TOSQ, TOBA, and TOAR thylakoid membranes denature in very similar ways. This is reassuring in indicating that the genetic manipulation affected only the fatty acid composition of the thylakoids, as intended. (To illustrate the variability between plants grown at different times, two different series are shown in Figures 3A and 2C. That is the reason the extents of protein denaturing are somewhat different in Figures 3A and 2C, for the three tobacco-based plants as compared to each other at ~68 °C.)

Comparison of the temperature of onset of protein denaturing in Figure 2C and the temperature of onset of the high-temperature decline of the ***D*** component frequencies in Figure 2B reveals a coincidence between them (denoted with an arrow). Thus, a purely lipidic feature of the infrared spectrum could be correlated with a purely proteinic feature. Since these are far apart in the infrared spectrum, this correlation cannot be an artifact.

To be absolutely sure about this correlation, we compared the three different parent plants as well, i.e., the wild types of *Arabidopsis*, squash, and tobacco. Figure 5A illustrates the frequencies of the ***D*** components as a function of temperature, while Figure 5B gives the evolutions of the 1620 cm^{-1} protein denaturing marker bands. It can be seen that the temperatures of onset of protein denaturing vary from plant to plant. The temperature dependence and the magnitude of the protein denaturing also differ for the three plants. It is common, however, that for each plant the temperature of onset of protein denaturing correlates with the breakdown of the frequency of the ***D*** component ($\nu_{sym}CH_2$ vibration), as shown by the corresponding arrows between panels A and B of Figure 5.

DISCUSSION

Membrane Dynamics. An unexpected finding of this analysis of membrane dynamics, based on the systematic decomposition of the $\nu_{sym}CH_2$ band into an ordered (***O***) and a disordered (***D***) fatty acyl chain segment population, is that, in the presence of a considerable amount of ***O*** component, the frequency of the ***D*** component cannot increase until the ***O*** component has disappeared in a phase transition-like process with increasing temperatures (Figure 2A,B). This is surprising, since the ***D*** component frequency would be expected to increase continuously toward higher temperatures, reflecting increasingly more disordered fatty acid populations.

For an understanding of this phenomenon, it should be borne in mind that in our approach the decomposition of the $\nu_{sym}CH_2$ band into only two components (Figure 1B) is

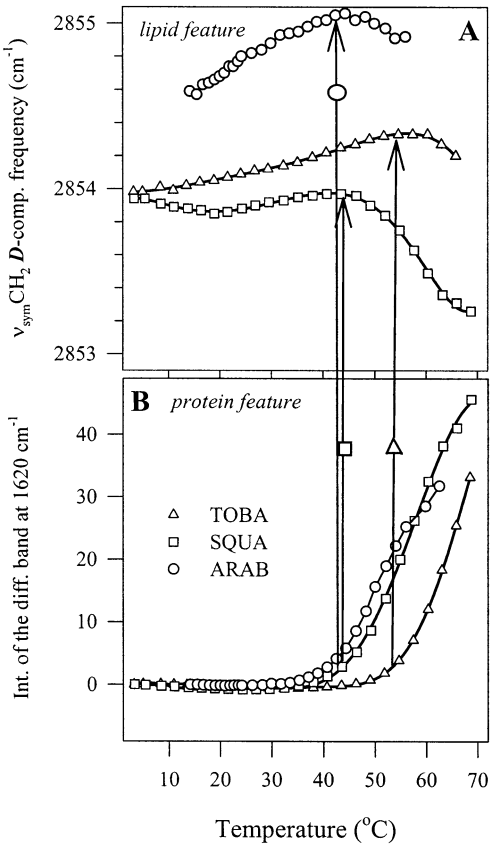

FIGURE 5: Correlation between lipid and protein dynamics in the thylakoid membranes of tobacco (TOBA), squash (SQUA), and *Arabidopsis* (ARAB). (A) $\nu_{sym}CH_2$ frequencies of the ***D*** population of the membrane lipid fatty acyl chains. (B) Heat-induced denaturing of the membrane proteins, as characterized by the increasing intensity of the difference band at ~1620 cm^{-1} (see Figure 4), assigned to intermolecular β-sheets formed upon protein aggregation. Arrows connect the corresponding lipid- and protein-related curves.

an approximation, and the signal-to-noise ratio and the number of data points do not allow more meaningful distinctions. As a further simplification of the situation, we assumed that molecules could be ordered only one way. Therefore, we did not consider any variability for the ***O*** component band. In contrast, the disordered state of the fatty acyl chains is not regarded as a well-defined, unique state. It represents a distribution over different molecular states, and the temperature can affect this distribution. The above strategy in the analysis was the fixing of the parameters of the ***O*** component, while those of the ***D*** component were left free when the spectra recorded as a function of temperature were fitted.

With these constraints in mind, the decreasing phase of the $\nu_{sym}CH_2$ frequency of the ***D*** component in TOSQ can be explained in the following way. At the lowest temperatures, the fatty acyl chains that are disordered are mostly those which interact directly with the proteins. Once the heat-induced ***O*** → ***D*** transition has taken place, fatty acyl chain segments pass from an ordered state to a disordered one, and the distribution of these freshly disordered segments over the possible conformations will not be the same as that in the protein-perturbed fatty acyl chains. The freshly "melted" distributed population will be "colder" (i.e., it will have a lower characteristic $\nu_{sym}CH_2$ frequency) than the *ab ovo* existing protein-related disordered lipid fatty acyl chain population. Since we fit only one band to the whole disordered population, the frequency of the ***D*** component can start to shift upward only after the ***O*** → ***D*** transition has been completed. As long as the melting of the ***O*** component supplies fresh disordered fatty acyl chains with lower $\nu_{sym}CH_2$ frequencies, the average $\nu_{sym}CH_2$ frequency of the overall ***D*** population will shift down, stagnate, or increase more slowly. These arguments together may prove that the two-component analysis of the $\nu_{sym}CH_2$ band can not only demonstrate the existence of two major CH_2 populations but also allow the changes within the distribution of the disordered fatty acyl chain segments to be followed to some extent.

The effect of the ***O*** component melting should be smaller in those plants where the ordered population is expected to be smaller. Indeed, whereas for TOSQ there is a spectacular decrease in the ***D*** component frequency up to ~25 °C, for TOBA only the rate of increase is somewhat lower up to this temperature and for TOAR no effect is visible at all (Figure 2B). In this transformant, the amount of *cis*-unsaturated fatty acids in PG is increased as compared with that in TOBA. [In SQUA, with more saturated fatty acids than in TOBA, a slight decrease in the ***D*** component frequency can be seen up to ~20 °C (Figure 5A).]

The magnitude of the difference between the ***O***/***D*** ratios of the wild-type and transformed plants seems at first glance to be too large, especially that between TOBA and TOSQ. Since PG accounts for only ~6−8% of the total phospholipids, changes in the fatty acid composition involve only 3−4% of the total fatty acid content. Nevertheless, we found this difference consequently in several series of experiments, and even the amplitudes of the differences were similar. Further, if the fact that the introduction of the squash GPAT into the tobacco increases the amounts of 16:0 palmitic acid and 18:0 stearic acid at the expense of the 18:1, 18:2, and 18:3 *cis*-unsaturated fatty acids is taken into account (*21*), the effect on the membrane dynamics should be larger than proportional to the fractions of the affected fatty acids. In themselves, palmitic and stearic acids would be in the gel phase, and the 18:1, 18:2, and 18:3 *cis*-unsaturated fatty acids in the liquid-crystalline phase at 5 °C. Additionally, saturated fatty acids present in larger amounts in a membrane may either form separate gel patches or exert a synergic effect on the order of the fatty acyl chains of the other lipids too.

Protein Structure. Up to ~40−45 °C, the average structures of the thylakoid membrane proteins are very similar in TOSQ, TOBA, and TOAR. These are basically the same plants, and therefore, this similarity supports other observations leading to the conclusion that the genetic manipulation of the tobacco with GPAT cDNAs affected only the fatty acid composition of the thylakoid membranes. The temperature courses of heat denaturing of the proteins of the thylakoid membranes were also very similar in the three tobacco-based plants (Figure 2C).

It is interesting that, despite the presence of squash-like PG molecules in TOSQ, the heat stability of the proteins in TOSQ did not shift down to become similar to that of SQUA, for which the temperature of onset of heat denaturing of its proteins was ~10 °C lower than that for TOBA (Figure 5B). This might be due to several reasons. (i) PG is not in such intimate contact with the proteins that it could influence their conformation or stability directly. Therefore, other, non-

manipulated lipids are probably responsible for the different thermal stabilities of the proteins in squash and tobacco. (ii) There are inherent differences between the proteins of SQUA and TOBA with regard to their structures and stabilities. This is less probable, since the amide I bands of the two plants are very similar at 5 °C (Figure 3B).

In the three tobacco-based plants, the heat stabilities of the proteins are identical (Figure 2C), despite the different fatty acid compositions of their PG molecules. This may mean that other lipids are indeed responsible for the maintenance of membrane and protein stability. At the same time, however, PG molecules may have the important role of "filling up" fragmented places at the protein−lipid interface; the small size and the charge of their headgroup would make such a role feasible. This function can be affected selectively and to a large extent by introducing genetically manipulated PG molecules into an optimized system.

The role of PG molecules in the organization and functioning of thylakoid membranes has been studied, and the findings connect these molecules to the functioning of the photosystem II (PSII) reaction center. By means of phospholipase C treatment that removed the headgroup of the phospholipid molecules, Droppa et al. (*26*) were able to decrease the PSII activity considerably. Kruse et al. (*27*) reported that PG molecules are involved in the dimerization of PSII. In cyanobacteria, Gombos et al. (*28*) have shown that the presence of PG molecules in the thylakoid membrane is necessary for the functioning of the electron acceptor plastoquinone Q_B in the PSII reaction center. However, in none of these works were the stability and dynamics of the involved protein components studied. Our conclusion that PG molecules are probably in close but "noninvasive" contact with the proteins, and thus participate in the fine-tuning of the working conditions of the proteins, fit well into the picture indicated so far by biochemical methods.

Lipid−Protein Interaction. The facts that protein denaturing involves major rearrangement in the thylakoid membranes affects lipid disorder are illustrated in panels B and C of Figure 2 and panels A and B of Figure 5. With regard to lipids, the second downshift of the ***D*** component frequencies at ∼2854 cm^{-1} (Figures 2B and 5A) correlates well with the temperature of onset of the evolution of the 1620 cm^{-1} band (shown in Figures 2C and 5B) related to protein denaturing and assigned to the formation of intermolecular β-sheets. If proteins aggregate, a smaller surface will be available for protein−lipid interactions, and therefore, some lipids will have to dissociate from the proteins. Once in their own phase, these lipids may form domains in which they are less disordered than they were at the protein−lipid interface. This means a lower average frequency for the ***D*** lipid component, with the frequency downshift starting at around the same temperature as that for protein denaturing (Figures 2B and 5B).

Consequences of Insertion of the Squash GPAT Gene into Tobacco. We observed differences between TOSQ and TOBA only in those parameters which were related to the membrane dynamics, and only at temperatures lower than 25 °C. Accordingly, we suggest that differences should be found between the two plants only under conditions that involve temperatures considerably lower than 25 °C. Indeed, Moon et al. (*21*) detected differences between tobacco and its squash GPAT-transformed mutant only in the sensitivity to low-temperature photoinhibition and in the speed of recovery from the photoinhibited state. Their low-temperature photoinhibition experiments involved the exposure of the plants to 1 °C, where the dynamic properties of the TOSQ and TOBA thylakoids are considerably different (Figure 2A). The extent of photoinhibition was larger and the rate of recovery slower in the squash cDNA-transformed TOSQ mutant with more rigid thylakoid membranes at low temperatures than in TOBA (Figure 2A). Thus, processes involving larger-scale rearrangements in the thylakoid membrane, such as insertion and processing of the D1 protein (regarded as crucial steps in the recovery process), most probably encounter more difficult conditions in the transgenic plant, and this is the reason for the lower recovery (*21*). There is a recent example in the literature for rice that supports the role of saturation and/or unsaturation of PG discussed above. By manipulating the membrane dynamics in the opposite direction as we did in this work, i.e., increasing the level of unsaturation of fatty acids in PG via transformation with *Arabidopsis* or spinach GPAT cDNAs, we increased the rate of photosynthesis and growth at low temperatures in the transgenic rice seedlings (*29*).

In conclusion, the introduction of PG molecules via the transformation of tobacco with squash GPAT cDNA led to measurable changes only in the dynamics of the membrane lipids, and only in the low-temperature region (5−25 °C); the membrane proteins were not affected. The PG molecules of transgenic origin probably accumulate at the protein−lipid interfaces of the thylakoid membrane of the transformed plant. Therefore, any physiological consequence of the squash GPAT gene expression in tobacco should be related to processes depending on lipid dynamics in the low-temperature range. This study demonstrates the power of the systematic analysis of FTIR spectra and, as far as we know, is the first such detailed investigation of the structural consequences of a genetic manipulation in a biological membrane, an approach that might also be useful in several other applications.

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SUPPORTING INFORMATION AVAILABLE

Differences between the one- and two-component analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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