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Commentary

Direct tests of the role of membrane lipid composition in low-temperature-induced photoinhibition and chilling sensitivity in plants and cyanobacteria

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Many plant species are injured or killed by exposure to low nonfreezing temperatures in the range of 1–12°C, whereas others may tolerate temperatures below 0°C without exhibiting deleterious effects (1). Cotton, soybeans, cucumbers, and squash are examples of chilling-sensitive plants, whereas wheat and *Arabidopsis thaliana* are examples of chilling-tolerant species. Because of the pronounced effects of temperature on the *in vitro* phase properties of lipids and the observation that membrane lipid composition may be altered in response to changes in growth temperature, there has been sustained interest in the possible role of variation in membrane lipid composition as a causal factor in chilling sensitivity. Although it has not been possible to establish simple correlations between total membrane lipid composition of different plant species and chilling sensitivity (2), interest in the concept has been sustained by the possibility that the amount of a quantitatively minor constituent might have a major effect on the physical properties of one or more membranes. Recently, the isolation of mutants of plants and cyanobacteria with specific alterations in membrane lipid composition and the isolation of some of the genes that control membrane lipid fatty acid composition have created new opportunities to directly test this and related concepts concerning the physiological importance of membrane lipid composition (reviewed in ref. 3). As exemplified by the article by Moon *et al.* (4) in this issue of the *Proceedings*, these lines of inquiry have recently produced several important new insights into the role of lipid composition in potentiating low-temperature-induced injury and have laid a new foundation for investigating the mechanistic basis for some aspects of chilling injury.

Role of Disaturated Phosphatidylglycerol (PG)

Murata and colleagues (5, 6) surveyed the molecular species of lipids present in chloroplast membranes from a variety of plant species representing both chilling-sensitive and chilling-resistant types. They observed a rough correlation between an *ad hoc* ranking of chilling sensitivity and the amount of PG containing only 16:0, 18:0, or 16:1-*trans* (*trans*-3-hexadecenoic acid) fatty acids. The fatty acid 16:1-*trans* has physical properties that are very similar to those of a saturated fatty acid (7). Thus, for convenience, the various molecular species of PG containing only these three fatty acids are collectively referred to as disaturated PG. PG typically constitutes 8–10% of chloroplast lipids. In chilling-resistant species typically less than about 20% of PG is disaturated, whereas in many chilling-sensitive species disaturated PG makes up more than 40% of chloroplast PG (8–10). Preparations of PG from several chilling-sensitive plants underwent the liquid-to-gel phase transition at approximately 30°C, whereas PG purified from chilling-resistant species underwent the transition below 15°C (11). Of the seven major leaf glycerolipid classes, only PG exhibited a significant phase separation, irrespective of whether the lipids were from chilling-sensitive or chilling-tolerant species. Furthermore, addition of as little as 2% or less of disaturated PG to mixtures of leaf membrane polar lipids significantly increased the temperature at which phase transitions occurred in the mixture (12). Thus, Murata and collaborators (5, 6) proposed that disaturated PG had a causal role in chilling sensitivity by causing a chilling-induced phase transition in chloroplast membranes.

Since chloroplast PG always contains 16:0 or 16:1-*trans* at the *sn*-2 position (6, 8), it was inferred that it was the substrate selectivity of the *sn*-1 glycerol-3-phosphate acyltransferase that was responsible for the interspecies variability in disaturated PG. This was an attractive hypothesis because it implied that variation in the properties of a single enzyme was responsible for chilling sensitivity. However, critics noted that the apparent correlation between the amount of disaturated PG and chilling sensitivity was not unambiguous because of the lack of a quantitative measure for chilling sensitivity. Also, in some chilling-sensitive species the total amount of 16:0 + 18:0 + 16:1-*trans* in PG, which has generally been assumed to be proportional to the amount of disaturated PG (8), is similar to the amount of disaturated PG in chilling-tolerant species (7). Thus, it has been inferred that disaturated PG is not a principal cause of chilling sensitivity in all species.

To test the disaturated-PG hypothesis, Murata and colleagues (13) purified the plastid glycerol-3-phosphate acyltransferase from squash and cloned the corresponding cDNAs from a chilling-sensitive species (squash) and a chilling-resistant species (*Arabidopsis*). They then placed the cDNAs under transcriptional control of a strong constitutive promoter and produced transgenic tobacco plants that expressed these genes (13). Transgenic plants that expressed the squash cDNA contained elevated levels of disaturated PG (76%), whereas plants that expressed the *Arabidopsis* cDNA contained slightly reduced levels of disaturated PG (28%) compared with wild-type tobacco (36%). Illumination of the control and transgenic plants with light at an intensity equivalent to full sunlight at 1°C for 4 h caused loss of photosynthetic capability to various degrees in the three classes of plants—a phenomenon that is generally designated low-temperature-induced photoinhibition (14, 15). The severity of photoinhibition was positively correlated with the levels of disaturated PG. Similarly, when plants were incubated at 1°C for 10 days in moderate light intensity (≈7% of the intensity of sunlight) the amount of visible injury due to chlorosis and necrosis of leaf tissue was positively correlated with the amount of disaturated PG. These results represented the first direct evidence for a causal link between the amount of disaturated PG and low-temperature-induced injury.

In a similar experiment, Wolter *et al.* (16) restructured the *plsB* gene for a membrane-bound glycerol-3-phosphate acyltransferase from *Escherichia coli* so that the polypeptide was directed into chloroplasts of transgenic *Arabidopsis* plants that expressed the gene. Since the bacterial enzyme preferentially utilizes 16:0 as a substrate, the transgenic lines contained increased levels of disaturated PG (48–54%) and slightly increased 16:0 in other glycerolipids. Only two transgenic plants were obtained, but they exhibited chilling-induced injury after 7 days at 4°C. Al-

though these results are consistent with those obtained by Murata *et al.* (13), the authors noted that interpretation of these experiments was somewhat complicated by the unknown effects of accumulation of the *plsB* protein in chloroplast membranes. Thus, it is possible that insertion of the bacterial polypeptide into chloroplasts membranes had a nonspecific deleterious effect that may have been exacerbated at low temperature.

In an article in this issue of the *Proceedings*, Moon *et al.* (4) have examined the basis for the chilling-induced photoinhibition in transgenic tobacco lines that contain increased amounts of disaturated PG. They found that there is no difference between the rate at which transgenic and wild-type plants undergo chilling-induced photoinhibition. Rather, the principal effect of the variation in the amount of disaturated PG seems to be on the rate at which damaged photosystems can be repaired. The main target for photoinhibition is thought to be the D1 polypeptide of the photosystem II reaction center (17, 18). When this protein is damaged, presumably by side products from the photochemical reactions (18), newly synthesized D1 protein is inserted into the photosystem II complex to restore photochemical activity. Thus, Moon *et al.* (4) hypothesize that the altered amount of disaturated PG has an effect on the rate at which damaged D1 protein is removed from the photosystem II complex and replaced by synthesis and insertion of new protein. An important unanswered question is whether the effect of disaturated-PG content on D1 turnover extends to other chloroplast membrane proteins. Conceivably, the D1 protein is simply an efficient reporter of a general defect in the assembly or removal of membrane proteins.

Independent evidence concerning the role of disaturated PG has been obtained from analysis of a mutant of *Arabidopsis* that has an increase in the amount of disaturated PG (19). Although the biochemical basis for the effect is not known, the *fab1* mutant of *Arabidopsis* has approximately 43% disaturated PG versus about 9% in wild-type *Arabidopsis*. In this respect, the *fab1* mutant has higher levels of disaturated PG than approximately half of all chilling-sensitive species that have been examined (5–8). When subjected to growth for 7 days at 2°C in the light levels normally used for growth (≈7% of full sunlight), the mutant *Arabidopsis* plants did not show any visible injury or deleterious effects and were indistinguishable from the wild type. By contrast, chilling-sensitive species such as cucumber, mung beans, green bean, green pepper, and castor bean were killed or severely damaged by these treatments. However, when grown for 28 days at 2°C in constant light, the mutant grew more slowly than the wild type and became chlorotic. These results seen to be at variance with those of Wolter *et al.* (16), who observed visible damage after a week at 4°C. This raises the possibility that the low-temperature-induced injury observed by Wolter *et al.* (16) may not be specifically due to disaturated PG or that the proportion of disaturated PG in the *fab1* mutant, which is less than that obtained by Wolter *et al.* (16), is below a critical threshold level needed to cause rapid chilling injury.

Although the *fab1* mutant does not exhibit the relatively rapid chilling injury observed in transgenic plants by Murata *et al.* (13) or Wolter *et al.* (16), the results of Wu and Browse (19) confirm a deleterious effect of high levels of disaturated PG on low-temperature fitness and provide a rationale for the relatively low content of disaturated PG among chilling-tolerant species which may be exposed to low temperatures for extended periods of the life cycle. Indeed, the chilling-induced chlorosis and slow growth of the *fab1* mutant could be due to a defect in chloroplast membrane protein turnover or accumulation of the kind described by Moon *et al.* (4). However, the side-by-side comparison of the *fab1* mutant with naturally chilling-sensitive species such as squash and cucumber that contain similar levels of disaturated PG (6, 9) emphasizes the point that factors other than high levels of disaturated PG are responsible for the chilling injury sustained by these and the other species tested by Wu and Browse (19). Thus, even though the deleterious effects of disaturated PG seem indisputable, the question remains as to what extent chilling sensitivity can be improved in species other than tobacco by decreasing the levels of disaturated PG. Will other cellular targets for chilling injury mask the beneficial effects of genetically modifying the amount of disaturated PG?

The Role of Fatty Acid Polyunsaturation

Several other recent studies are generally consistent with the concept that membrane lipid unsaturation can have a strong effect on membrane biogenesis that is superficially similar to the effects of disaturated PG. Direct evidence for the importance of general membrane polyunsaturation in recovery from low-temperature-induced photoinhibition was observed in a mutant of *Synechocystis* PCC6803 that was deficient in polyunsaturation (20). In these experiments, there was no difference between the mutant and the wild type in the rate of photoinhibition at low temperature, but the rate of recovery of photosynthetic competence was significantly lower in the mutant at low temperature. Thus, it was concluded that polyunsaturation of glycerolipids facilitates recovery from photoinhibition. It is notable that the conclusion from these experiments was virtually identical to that from the experiments described by Moon *et al.* (4) in which the amount of disaturated PG was altered. These observations suggest that the effects of high levels of disaturated-PG may be similar or identical to the effects of proportionately larger decreases in overall membrane polyunsaturation rather than to a unique contribution of disaturated-PG *per se*.

A proposal that membrane polyunsaturation is required for membrane biogenesis at low temperature was also previously made to explain the observation that several mutants of *Arabidopsis* with defects in chloroplast membrane polyunsaturation were chlorotic and deficient in chloroplast membranes when grown at low temperature. Tissues that had developed before transfer to low temperature were not visibly affected by prolonged exposure to low temperature, indicating that membrane integrity was not compromised. By contrast, tissues that developed at low temperature were chlorotic because they contained as little as 25% as much chloroplast membrane as wild type. Thus, an effect on membrane biogenesis rather than function was proposed to be the primary consequence of reduced levels of polyunsaturation (21). It now seems likely that these mutants may be expected to also exhibit chilling-induced photoinhibition under the conditions used to test chilling injury in tobacco (4, 13).

The *fad2* mutants of *Arabidopsis* have a defect in nonplastid membrane lipid polyunsaturation due to the loss of activity of an endoplasmic reticulum-localized oleate desaturase (22). *fad2* mutants are unable to grow at temperatures as high as 6°C but do not exhibit any visible symptoms of chilling-induced injury during the first week or more following exposure to temperatures as low as 1°C (23). The long delay in appearance of any signs of chilling injury indicates that the phenotype is due not to an immediate low-temperature-induced disruption of membrane function but rather to a chronic effect that is expressed only during biogenesis or maintenance of membranes.

During the past several years most of the fatty acid desaturases from higher plants and cyanobacteria have been cloned (refs. 22 and 24–27; partially summarized in ref. 28). The availability of these genes has created new opportunities to alter the composition of membranes and to examine the mechanisms that control membrane lipid composition. In this respect, Kodama *et al.* (29) have expressed a chloroplast-localized $\omega - 3$ desaturase from *Arabidopsis* in transgenic tobacco. The transgenic plants contained increased levels of trienoic fatty acids (16:3 + 18:3) and correspondingly decreased levels of less unsaturated species. The transgenic plants exhibited a significantly higher leaf expansion rate than control plants follow-

ing a 7-day exposure to 1°C. In addition, the transgenic plants exhibited less visible damage during the period of low-temperature exposure. This is a particularly intriguing result because such a change in lipid composition would not be expected to affect the tendency of chloroplast lipids to undergo a phase transition at temperatures above 0°C. These results also suggest that it may be possible to incrementally improve chilling tolerance of some species by combining several engineered traits such as decreased disaturated PG and increased polyunsaturation.

The Regulation of Membrane Unsaturation

Many plant species change the extent of membrane lipid unsaturation in response to alteration in growth temperature (30). As a general rule, the level of desaturation decreases as growth temperature increases. This observation, by itself, may be considered evidence that regulated variation in lipid composition is advantageous in adapting to different temperature conditions. Knowledge of the mechanisms that lead to temperature-induced changes in membrane composition may provide insights into the parameters of membrane structure or function that are perceived by the organism as most critical.

A first glimpse of the underlying phenomena has been provided by studies of the effects of temperature on expression of the 12-desaturase from *Synechocystis* PCC6803 encoded by the *desA* gene. The level of the *desA* transcript increased 10-fold within 1-hr after a decrease in the growth temperature from 36°C to 22°C (31). Thus, in cyanobacteria, membrane composition is controlled at least in part at the transcriptional level. The essential question that arises is whether transcription of the gene is regulated by mechanisms that respond directly to temperature or by mechanisms that sense a physical property of the membrane. An insight into this problem was elegantly obtained by using catalytic hydrogenation to decrease the degree of plasma membrane fatty acid unsaturation in living cells of *Synechocystis* PCC6803 (32). Following a brief period of hydrogenation, the level of the *desA* transcript was strongly increased, indicating that the level of the *desA* transcript is regulated in response to changes in the physical properties of the plasma membrane. A similar effect has been observed in yeast, where expression of the stearoyl-CoA desaturase gene, *ole1*, was depressed by feeding unsaturated fatty acids (33).

Somewhat surprisingly, the available evidence suggests that higher plants do not have a similar mechanism for regulating expression of desaturases in response to changes in the physical properties of membranes. Of the six different higher plant desaturases for which genes are available, only the *Arabidopsis fad8* gene, a plastid 15-desaturase, exhibits significant changes in transcript accumulation in response to a change in growth temperature (27). However, the levels of *fad8* transcript are not increased in mutants that are deficient in trienoic fatty acids due to mutations in the *fad8* gene and an isozyme encoded by the *fad7* gene as would be expected if the level of *fad8* transcript was regulated in response to the physical properties of the chloroplast membranes. A similar conclusion may be drawn from the fact that heterozygotes for most of the fatty acid desaturase-deficient mutants of *Arabidopsis* have levels of membrane lipid fatty acid desaturation that are intermediate between those of the mutant and the wild type (34). Thus, the available evidence suggests that in plants the regulation of the lipid compositions of the various intracellular membranes may take place at the posttranscriptional level. Perhaps this apparent difference between modes of regulation in plants and cyanobacteria is related to the fact that eukaryotic cells must regulate the properties of many membranes with different structures and functions, whereas cyanobacteria have fewer membranes.

Concluding Remarks

In conclusion, molecular genetic approaches have provided powerful new tools for investigating and testing the mechanisms that regulate membrane lipid fatty acid composition and for genetically improving the chilling tolerance of chilling-sensitive species. Direct tests of the role of membrane composition have substantiated decades of physiological studies suggesting a role for lipid composition in at least some instances of chilling-induced injury. The work of Moon *et al.* (4) and similar work by Gombos *et al.* (20) represent an important step toward defining a specific biological function that is directly affected by several different kinds of changes in membrane lipid composition. It is not immediately obvious how, or if, the apparent role of lipid composition in facilitating membrane protein assembly or turnover is related to the *in vitro* lipid phase changes that originally stimulated interest in the possible role of membrane lipids. However, such a role might explain the kinds of phenotypes observed in mutants with decreases in membrane polyunsaturation. Finally, as with any genuine advance, the results of Moon *et al.* (4) suggest many potentially informative new experiments.

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