# Genetic Engineering of Chilling Sensitivity

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## MEMBRANE LIPIDS AND CHILLING SENSITIVITY

Lyons [Lyons, 1973] and Raison [Raison, 1973] proposed that the primary event in chilling injury is the formation of a lipid gel phase in cellular membranes as temperature decreases, which is followed by a series of processes leading to the death of the cells. When a model membrane goes into the phase-separated state, in which both gel and liquid crystalline phases coexist, the membrane becomes permeable to small electrolytes, thereby diminishing membrane ion gradients that are essential for maintenance of cellular activities [Murata & Nishida, 1990].

Higher-plant cells contain monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and phosphatidylglycerol (PG) in plastid membranes; phosphatidylcholine (PC), phosphatidylethanolanine (PE), diphosphatidylglycerol (DPG), and PG in mitochondrial membranes and PC, PE, and phosphatidylinositol (PI) in endoplasmic reticulum and plasma membranes [Murata & Nishida, 1990]. The temperature for the transition between the gel and liquid crystalline phases of glycerolipid molecular species varies markedly with the level of unsaturation of the fatty acyl chains. The molecular species containing only saturated fatty acids, such as 16:0 and 18:0, reveal phase transition temperatures above 40°C. The molecular species containing a *cis*-unsaturation bond reveal phase transition near 0°C [Phillips et al, 1972]; and the introduction of the second *cis*-unsaturation bond decreases the phase transition temperature to about -20°C [Phillips et al, 1972]. The substitution of 16:0 by *trans*-monounsaturated fatty acid, 16:1t, at the C-2 position of PG, shifts the phase transition temperature by 10°C [Kenrick & Bishop, 1986]. These findings suggest that if certain lipid molecular species can alter membrane phase transition above 0°C, they are most likely to be the fully saturated or *trans*-monounsaturated species. In all glycerolipids from leaf cells, only PG contains high levels of these molecular species [Murata et al, 1982; Murata, 1983; Raison & Wright, 1983; Murata & Yamaya, 1984].

In a previous study, we demonstrated that chilling-sensitive plants contain much higher proportions of the 16:0/16:0 plus 16:0/16:1t species of PG than do chilling-resistant plants [Murata et al, 1982]. In about 20 plants examined in our laboratory, these molecular species represented from 3 to 19% of the total PG in the chilling-resistant plants, and from 26 to 65% in the chilling-sensitive plants [Murata, 1983]. Additionally, Roughan [Roughan , 1985] surveyed the fatty acid composition of PG in 74 plants, and found a correlation between chilling sensitivity and the level of saturated plus *trans*-monounsaturated PG molecular species. Taken together, these findings strongly suggest that these two molecular species are closely associated with the chilling sensitivity of plants.

## TARGET GENE: GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE

On the basis of the knowledge concerning the biosynthesis of PG in plastids [Andrews & Mudd, 1985], we have suggested that the level of saturated and *trans*-unsaturated PG depends on the substrate selectivity of the first step of PG synthesis, i.e. the transfer of the acyl group from acyl-acyl-carrier protein (ACP) to the C1 position of glycerol 3-phosphate [Murata, 1983]. The enzyme for this reaction is called acyl-ACP: glycerol-3-phosphate acyltransferase (hereinafter acyltransferase), and is local in the stroma of chloroplasts [Frentzen et al, 1983]. In fact, the substrate selectivity of acyltransferases from chilling-resistant plants is preferential to *cis*-unsaturated fatty acids, whereas this enzyme in chilling-sensitive plants cannot discriminate saturated and *cis*-unsaturated fatty acids [Frentzen et al, 1987; Nishida et al, 1987]. Thus, genetic engineering for this enzyme should enable us to convert plants from chilling-sensitive to chilling-resistant, and *vice versa*. Since cDNAs for acyltransferase have been cloned from a chilling-sensitive plant, squash [Ishizaki et al, 1988], and a chilling-resistant plant, *Arabidopsis thaliana* [Nishida et al, 1993], it is now possible to change the level of fatty acid unsaturation of PG, and thus the chilling sensitivity, in higher plants such as tobacco [Murata et al, 1992].

## TRANSFORMATION OF TOBACCO

Ti-plasmids for over-expressing the cDNA for acyltransferases of squash [Ishizaki et al, 1988] and *Arabidopsis* [Nishida et al, 1993] in tobacco plants were constructed in pBI-121 under the control of the cauliflower mosaic virus 35S

constitutive promotor. The GUS region of pBI-121 was replaced by a cDNA insert containing the full-length coding sequence for a precursor to *Arabidopsis* acyltransferase and its 5'- and 3'-noncoding regions [Nishida et al, 1993]. This plasmid is designated pARA. In another construct, the GUS region of pBI-121 was replaced by a fusion construct in which the mature-protein region of cDNA for squash acyltransferase [Ishizaki et al, 1988] was ligated with the transit peptide region of cDNA for the small subunit of pea Rubisco [Schreier et al, 1985]. This plasmid is designated pSQ. The Ti-plasmids were introduced into *Agrobacterium tumefaciens* by electroporation, and the resultant transformants were selected by resistance to kanamycin and by DNA-DNA hybridization analysis for integrated cDNA sequences. *Nicotiana tabacum* var. Samsum was transformed by a leaf-disk method.

Fifteen independent clones were arbitrarily chosen from each group of transgenic tobacco plants to examine the level of unsaturation in PG from the leaves. Most of the transformants showed changes in the level of PG unsaturation. From the fifteen transformants of each group, five clones, which showed large changes in unsaturation, were selected for further analysis.

DNA-DNA hybridization analysis of genomic DNA from these transgenic tobacco plants indicated that the introduced cDNAs were stably integrated into the genome. The number of integrated cDNAs was estimated to be in the range of 1 to 5 copies per genome. RNA-DNA hybridization analysis of poly(A)$^+$ RNA from leaves of the transformants indicated that the message was expressed at high levels. Immunoblot analysis using antibodies against acyltransferases from *Arabidopsis* and squash revealed that, in the transformants with the *Arabidopsis* and squash cDNAs, mature proteins were detected in both the total protein extracts from leaves and in the soluble protein extracts from isolated chloroplasts. The levels of the over-expressed proteins were estimated to range from 0.1% to 1% of the total leaf proteins. This is much higher than the endogenous level of acyltransferase in wild-type tobacco plants. These observations demonstrate that the over-expressed proteins are transported into chloroplasts where they are then processed to mature proteins.

## ASSESSMENT OF TRANSGENIC PLANTS

An analysis of the lipids from leaves of the transgenic tobacco plants revealed no discernible change in the overall fatty-acid compositions between the transformed and the non-

transformed plants. Analysis of individual lipid classes revealed that PG was the only lipid class whose fatty-acid unsaturation level was significantly altered by transformation with cDNAs for acyltransferase (Table I). When the squash cDNA with pSQ was introduced, the level of saturated and *trans*-unsaturated PG increased from 36% to 76%. In contrast, the introduction of the *Arabidopsis* cDNA with pARA caused a small but significant decrease in these molecular species, i.e., from 36% to 28%.

**Table I.** *Changes in unsaturation of PG of tobacco by transformation with cDNA for acyltransferases from squash and Arabidopsis (calculated from data in [Ishizaki et al, 1988])*

| Transgenic plant | Molecular species | | | | |
|---|---|---|---|---|---|
| | 16:0 16:0/16:1t PG | 18:0 16:0/16:1t PG | 18:1 16:0/16:1t PG | 18:2 16:0/16:1t PG | 18:3 16:0/16:1t PG |
| | (% of molecular species) | | | | |
| Wild type | 32 | 4 | 8 | 22 | 34 |
| Transgenic with | | | | | |
| pBI121 (control) | 34 | 2 | 12 | 22 | 30 |
| pSQ | 62 | 14 | 4 | 6 | 14 |
| pARA | 24 | 4 | 14 | 24 | 34 |

Abbreviations: 16:0, hexadecanoic acid (palmitic acid); 16:1t, Δ3-trans hexadecenoic acid; 18:0, octadecanoic acid (stearic acid); 18:1, Δ9-octadecenoic acid (oleic acid); 18:2, Δ9,12-octadecadienoic acid (linoleic acid); 18:3, Δ9,12,15-octadecatrienoic acid (α-linolenic acid).

The transgenic plants were then analyzed for chilling sensitivity. The transformant with pBI-121 and wild-type tobacco showed some chilling sensitivity, but upon the introduction of the cDNA for squash acyltransferase, this sensitivity increased markedly. By contrast, the introduction of the cDNA for *Arabidopsis* acyltransferase decreased chilling sensitivity [Murata et al, 1992].

Exposure of whole plants to a temperature of 1°C for 10 days produced a markedly different appearance of the leaves among the transformants with different constructs. These changes included varying degrees of chlorosis and leaf deterioration. This damage appeared more clearly when the plants were kept at 25°C for 2 days after the chilling treatment. Leaves of the wild-type tobacco and the transformants with pBI-121 showed only partial chlorosis, while the leaves of the transformants with pSQ were severely damaged or completely dead. Further, the transformants with pARA were much more resistant to chilling than the wild-type tobacco or the transformants with pBI-121. These differences in chilling sensitivity correlated well with the level of fatty-acid unsaturation of PG in these plants.

Heinz and his collaborators [Wolter et al, 1992] performed a similar experiment with a gene from *E. coli*. They introduced, into *Arabidopsis thaliana*, an *E. coli* gene for acyltransferase [Lightner, 1983] which preferentially transfers saturated fatty acids to the C-1 position of glycerol-3-phosphate. This transformation increased the level of saturated plus *trans*-monounsaturated PG, and converted *A. thaliana* into a chilling-sensitive plant.

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