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# Chilling sensitivity of *Arabidopsis thaliana* with genetically engineered membrane lipids

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**Upon transfer of a genetically engineered *Escherichia coli* gene for glycerol-3-phosphate acyltransferase (*plsB*) to *Arabidopsis thaliana* (L.) Heynh., the gene is transcribed and translated into an enzymatically active polypeptide. This leads to an alteration in fatty acid composition of membrane lipids. From these alterations it is evident that the enzyme is located mainly inside the plastids. The amount of saturated fatty acids in plastidial membrane lipids increased. In particular, the fraction of high-temperature melting species of phosphatidylglycerol is elevated. These molecules are thought to play a crucial role in determining chilling sensitivity of plants. An increase in sensitivity could be observed in the transgenic plants during recultivation after chilling treatment. Implications for the hypothesis of phosphatidylglycerol-determined chilling sensitivity are discussed.**

*Key words:* acyltransferase/fatty acid composition/phosphatidylglycerol/stress/transgenic plant

## Introduction

Many plants of tropical and subtropical origin, when exposed to low non-freezing temperatures (0-15°C), develop symptoms indicative of injured performance that are collectively named chilling sensitivity. The critical temperatures may interfere in many functions with consequences for growth, yield, transport and storage in such plants (Long and Woodward, 1988). The phenomenon always includes impairment exceeding that resulting from the reduction of metabolic rates caused by decreasing temperatures. The metabolic interference at low temperatures may also limit growth or survival after subsequent return to higher temperatures. Maintenance of a fluid state of membrane lipid components is thought to be one of the prerequisites for unimpaired survival at lower temperature (Lyons, 1973). A lateral phase separation of non-fluid membrane components may be the primary defect caused by exposure to low temperature.

Chilling sensitivity in different plants may be mediated by different membrane systems depending on their content of specific, non-fluid lipid classes. In plastidic membranes phosphatidylglycerol (PG) has been identified as a critical component in this respect (Murata, 1983). The correlation between chilling sensitivity and a high content of non-fluid PG has been exemplified in many plants (Roughan, 1985; Bishop, 1986). The phase transition of this high-temperature melting fraction of phosphatidylglycerol (htm-PG) is the first event to occur as temperature drops which then initiates membrane dysfunction (Raison and Wright, 1983; Murata and Yamaya, 1984; Bishop and Kenrick, 1987). On the other hand, a low proportion of htm-PG is required but not sufficient for chilling tolerance, since additional factors are essential for membrane function at low temperature (Long and Woodward, 1988).

In chilling tolerant plants, all these requirements are met and, therefore, defined factors can be experimentally perturbed. In the experiments described here, we have increased the proportion of saturated fatty acids in PG in chloroplasts and thus increased its non-fluid proportion. This caused a chilling tolerant plant to become chilling sensitive with symptoms visible during recultivation of cold-treated plants under warm conditions. In parallel work published recently (Murata *et al.*, 1992) a similar strategy was followed and despite the use of different plant species and enzymes both approaches came to the same conclusions and, therefore, provide independent evidence for the involvment of PG in chilling sensitivity.

The biosynthesis of the non-fluid PG component in plastids (Mudd *et al.*, 1987) can be attributed to the properties of a single enzyme, acyl-ACP:*sn*-glycerol-3-phosphate acyltransferase (GPAT) (EC 2.3.1.15). This acyltransferase initiates glycerolipid synthesis in plastids by catalysing the acylation of the C1 position of *sn*-glycerol-3-phosphate. In chilling tolerant plants, the acyltransferase selects only oleic acid (18:1) from the mixture of acyl-ACP containing mainly 18:1 and palmitic acid (16:0) (Frentzen *et al.*, 1983). In contrast, in chilling sensitive plants (Cronan and Roughan 1987; Frentzen *et al.*, 1987), the enzyme indiscriminately accepts all acyl groups of acyl-ACP. The product 1-acyl-glycerol-3-phosphate is subsequently esterified at C2 with 16:0 in both chilling tolerant and sensitive plants (Frentzen *et al.*, 1983). The resultant phosphatidic acid is converted to PG (Mudd *et al.*, 1987) in which the fatty acids reflect the selectivity of the first acyltransferase: chilling resistant plants produce mainly 18:1/16:0 PG, whereas chilling sensitive plants have in addition various proportions of 16:0/16:0 PG, depending on the selectivity of the particular acyltransferase. After assembly, C1-bound 18:1 is converted to linoleic (18:2) and further to linolenic acid (18:3). The C2-bound 16:0 is partially converted to 3-*trans*-hexadecenoic acid (*trans*-16:1), which in its physical properties is similar to a saturated fatty acid (Bishop and Kenrick, 1987; Murata and Yamaya, 1984; Raison and Wright, 1983). Therefore, it is the lack of selectivity of the first acyltransferase which results in the accumulation of high-temperature melting 16:0/16:0 and 16:0/*trans*-16:1 species of PG.

To increase the accumulation of htm-PG in a chilling tolerant plant, we transformed such plants in order to express

an acyltransferase that should increase the incorporation of 16:0 into the C1 position of glycerol-3-phosphate during lipid synthesis in chloroplasts. The acyltransferase from *Escherichia coli* encoded by the *plsB* gene (Lightner *et al.*, 1983) is well suited for this purpose, as it preferentially uses 16:0 for acylation (Rock *et al.*, 1981), and in fact, the transformation of a chilling tolerant plant with the *plsB* gene of *E.coli* resulted in increased chilling sensitivity.

## Results

### *Construction of a chimeric acyltransferase gene*

To achieve functional expression and correct subcellular targeting of the bacterial *plsB* gene we combined four different genetic elements. A strong plant promoter and the coding region of a signal sequence for import into plastids (transit peptide) were both isolated from rbcS-1, a strongly expressed gene of the small subunit of ribulose bisphosphate carboxylase (rubisco) of potato (Wolter *et al.*, 1988). This was fused to the coding region of the *plsB* gene from *E.coli* (Lightner *et al.*, 1983), and finally the polyadenylation signal from the octopine synthase gene was added (Koncz *et al.*, 1987). The fusion protein encoded by this chimeric construct starts with a transit peptide of 60 amino acids identical to that of rbcS-1 followed by the first three amino acids of the mature small subunit of rubisco. The following tryptophan codon specifies the fourth amino acid of the mature small subunit of rubisco as well as the fourth codon following the first of the two possible start codons of *plsB* (Lightner *et al.*, 1983). The *plsB* coding region continues for 803 codons.

### *Transformation of Arabidopsis*

The chimeric gene construct was cloned as a *Hin*dIII fragment into the unique *Hin*dIII site of pGSC1704 plasmid which was transferred to *Agrobacterium*. Cocultivation, selection and shoot induction of *Arabidopsis thaliana* were carried out as described earlier (Schmidt and Willmitzer, 1988). From two experiments six independent hygromycin resistant lines were regenerated. Plants of the fourth generation obtained by successive self-crossing were used for further analysis and in particular two lines, no.4 and no.6, were analysed in detail. Only plants from line no.4 exhibit a phenotype which differs significantly from wild type. These plants are tiny and prone to premature flowering. As a consequence no typical leaf rosette is formed and a multitude of shoots emerges from each plant. Plants of line no.6 are similar in habitus to wild type, but plants from both lines grow slowly and have a low yield of viable seeds.

### *Expression*

To demonstrate that the *plsB* sequence is actively transcribed total RNA was isolated from leaves of transgenic plants. After separation by gel electrophoresis and transfer to nylon filters the RNA was hybridized to a labelled probe of *plsB* antisense RNA (Figure 1). For both transgenic lines a signal of ~3 kb was observed, which is reproducibly more intensive in samples from line no.4. This size is in excellent agreement with the expected length for a transcript derived from the chimeric pHAMPL construct coding for 860 amino acids plus 5′ and 3′ non-translated regions.

To prove that this RNA is properly translated, proteins from crude membrane fractions were subjected to SDS−PAGE and transferred to nylon membranes. The *PlsB*

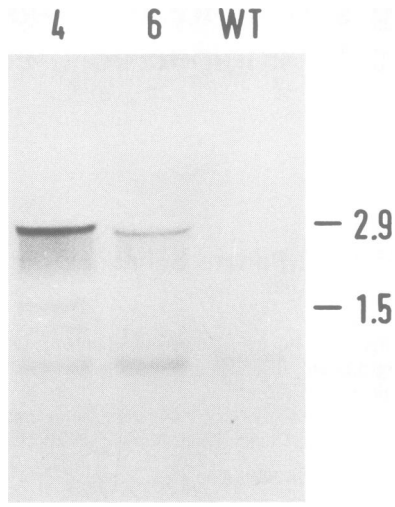


**Fig. 1.** Transcription of the bacterial acyltransferase gene in transgenic plants as analysed by Northern blotting. Identical quantities of total leaf RNA (15 μg) were subjected to electrophoresis, transferred to a nylon filter, and probed with a digoxigenin-labelled RNA complementary to a 1.2 kb fragment of the bacterial *plsB* gene. RNA from the transgenic lines no.4 and no.6 were loaded on to the left two lanes, RNA from wild type (WT) on to the right lane. The position and size of marker RNAs is indicated at the right.

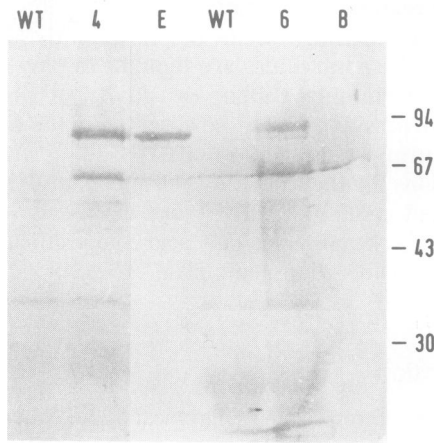


**Fig. 2.** Detection of the bacterial acyltransferase in transgenic plants by Western blotting. Identical quantities of membrane material corresponding to 20 μg chlorophyll were subjected to SDS−PAGE and probed with an antibody against the bacterial acyltransferase. Location and size of marker proteins are indicated at the right. WT, membranes from wild type plants; 4, membranes from transgenic line no.4; 6, membranes from transgenic line no.6; E, partially purified *E.coli* acyltransferase; B, sample buffer.

polypeptide was detected by anti-*PlsB* antibodies as shown in Figure 2. Lines no.4 and no.6 exhibit a positive reaction with the signal corresponding to a protein of ~90 kDa, which again agrees well with the expected molecular weight of the *plsB* protein. Weaker signals at lower molecular weight are probably due to degradation products.

Additionally, membrane fractions from wild type and transgenic plants were used for an enzymatic assay, and increased GPAT activity was observed in both transgenic lines. For line no.6 a 4-fold higher activity was measured as compared with control membranes, and in line no.4 the value was eight times higher. Therefore, we conclude that both transgenic lines transcribe the *plsB* gene and translate

**Table I.** Lipid composition of wild type (WT) and transgenic *Arabidopsis* plants (lines no.4 and no.6)

| Lipid component | WT | no.4 | no.6 |
|---|---|---|---|
| Monogalactosyl diacylglycerol (MGD) | 40 | 39 | 39 |
| Digalactosyl diacylglycerol (DGD) | 16 | 14 | 15 |
| Sulfoquinovosyl diacylglycerol (SQD) | 3 | 4 | 3 |
| Cerebroside | 2 | 3 | 2 |
| Steryl glucoside | 2 | 3 | 1 |
| Phosphatidylcholine (PC) | 16 | 15 | 16 |
| Phosphatidylethanolamine (PE) | 7 | 7 | 7 |
| Phosphatidylglycerol (PG) | 9 | 9 | 10 |
| Phosphatidylinositol | 4 | 5 | 5 |
| Phosphatidylserine | <1 | <1 | <1 |
| Phosphatidic acid | <1 | <1 | <1 |

Data are given as mole %.

**Table II.** Fatty acid composition of the main lipid classes for wild type (WT) and transgenic plants (lines no.4 and no.6)

| | | Fatty acid | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lipid | Plant | 16:0 | 16:1[a] | 16:2 | 16:3 | 18:0 | 18:1 | 18:2 | 18:3 |
| PG | WT | 34 | 21 | 0 | 1 | 2 | 8 | 8 | 26 |
| | no.4 | 56 | 17 | 0 | 1 | 4 | 3 | 4 | 16 |
| | no.6 | 53 | 17 | 0 | 0 | 4 | 3 | 5 | 17 |
| MGD | WT | 2 | 1 | 2 | 31 | 0 | 1 | 2 | 62 |
| | no.4 | 6 | 1 | 2 | 24 | 0 | 1 | 2 | 65 |
| | no.6 | 4 | 0 | 2 | 30 | 0 | 1 | 2 | 61 |
| DGD | WT | 16 | 0 | 1 | 2 | 2 | 2 | 4 | 73 |
| | no.4 | 23 | 0 | 1 | 1 | 3 | 1 | 3 | 69 |
| | no.6 | 30 | 0 | 1 | 2 | 3 | 1 | 2 | 60 |
| SQD | WT | 40 | 0 | 0 | 1 | 3 | 3 | 8 | 45 |
| | no.4 | 54 | 0 | 0 | 1 | 4 | 2 | 6 | 34 |
| | no.6 | 44 | 0 | 1 | 0 | 3 | 2 | 7 | 45 |
| PC | WT | 22 | 1 | 0 | 1 | 2 | 6 | 29 | 39 |
| | no.4 | 30 | 0 | 0 | 0 | 4 | 4 | 23 | 40 |
| | no.6 | 29 | 0 | 0 | 1 | 3 | 4 | 23 | 41 |
| PE | WT | 31 | 0 | 0 | 0 | 3 | 4 | 33 | 30 |
| | no.4 | 35 | 0 | 0 | 0 | 2 | 3 | 26 | 33 |
| | no.6 | 35 | 0 | 0 | 0 | 2 | 2 | 26 | 34 |

[a]16:1 in PG is *trans*-16:1.
For abbreviations see Table I. Fatty acids are characterized by carbon and double bond numbers. Data are given in mole %.

the RNA into an enzymatically active protein of the expected size. The expressed protein as well as the enzymatic activity are associated with membranes. The expression is higher in line no.4 as judged from Northern blots as well as from measured enzymatic activity.

### *Lipid composition*

The expression of an additional enzyme of bacterial origin which contributes to the synthesis of lipids, does not alter the overall proportions of membrane lipids in leaves, and in particular, the proportion of PG in chloroplasts was not significantly changed (Table I). A detailed analysis of the fatty acid composition in the different lipid classes and of their positional distribution in the main plastidial lipids revealed characteristic differences between the transgenic lines and wild type plants (Tables II and III). The data for wild type plants regarding lipid proportions as well as profiles and positional distribution of fatty acids are in good agreement with previously obtained figures (Browse *et al.*, 1986b, 1989a; Norman and St John, 1986; Kunst *et al.*, 1988, 1989). In transgenic plants the proportion of 16:0 is increased in all lipid classes to various extents. The increase is limited to the *sn*-1 position which is controlled by GPAT, and correlates with the proportion of the particular lipid class synthesized via the prokaryotic pathway within the plastid (Browse *et al.*, 1986a). Accordingly, the individual lipids show different changes in fatty acid profiles which will be detailed in the following with emphasis on the three major chloroplast lipids monogalactosyl diacylglycerol (MGD), digalactosyl diacylglycerol (DGD) and PG.

For MGD of line no.6 only small differences from wild type plants are seen which agrees with the weaker expression of the bacterial acyltransferase in these plants. In line no.4 a larger 16:0 increase was observed at *sn*-1, whereas in *sn*-2 C18 fatty acids were increased at the expense of C16 acids. This indicates an increase in eukaryotic MGD and may represent a compensatory effect. The additional 16:0 in *sn*-1 of MGD is not desaturated despite the fact that plants have this capacity as shown for spinach (Roughan *et al.*, 1987; Heemskerk *et al.*, 1991). As the increase in 16:0 is not

**Table III.** Positional distribution of fatty acids in the main plastidial lipid classes from wild type (WT) and transgenic plants (lines no. 4 and no. 6)

| Lipid | | Plant | 16:0 | 16:1[a] | 16:2 | 16:3 | 18:0 | 18:1 | 18:2 | 18:3 |
|---|---|---|---|---|---|---|---|---|---|---|
| PG | *sn-1* | WT | 25 | 0 | 0 | 0 | 5 | 16 | 15 | 38 |
| | | no.4 | 61 | 0 | 0 | 0 | 6 | 5 | 7 | 21 |
| | | no.6 | 54 | 0 | 0 | 0 | 7 | 6 | 8 | 25 |
| | *sn-2* | WT | 55 | 37 | 0 | 1 | 1 | 1 | 2 | 4 |
| | | no.4 | 55 | 32 | 0 | 1 | 2 | 1 | 3 | 6 |
| | | no.6 | 56 | 35 | 0 | 1 | 2 | 1 | 2 | 5 |
| MGD | *sn-1* | WT | 3 | 0 | 1 | 2 | 1 | 2 | 3 | 88 |
| | | no.4 | 11 | 1 | 2 | 3 | 1 | 1 | 2 | 79 |
| | | no.6 | 7 | 1 | 2 | 3 | 1 | 1 | 2 | 84 |
| | *sn-2* | WT | 2 | 3 | 2 | 58 | 0 | 1 | 2 | 32 |
| | | no.4 | 3 | 1 | 2 | 36 | 0 | 0 | 3 | 56 |
| | | no.6 | 2 | 1 | 2 | 53 | 0 | 0 | 1 | 40 |
| DGD | *sn-1* | WT | 15 | 0 | 2 | 1 | 4 | 3 | 5 | 70 |
| | | no.4 | 37 | 0 | 2 | 1 | 5 | 2 | 3 | 50 |
| | | no.6 | 43 | 0 | 2 | 1 | 5 | 2 | 3 | 44 |
| | *sn-2* | WT | 17 | 0 | 1 | 5 | 2 | 2 | 2 | 71 |
| | | no.4 | 19 | 0 | 2 | 5 | 2 | 1 | 2 | 69 |
| | | no.6 | 24 | 0 | 2 | 3 | 1 | 1 | 2 | 67 |

[a]16:1 in PG is *trans*-16:1.
For abbreviations see Table I. Data are given as mole %.

balanced by increased desaturation of other fatty acids in prokaryotic MGD, this more saturated component may be subjected to an increased turnover resulting in its replacement by highly unsaturated eukaryotic MGD. A similar switching to eukaryotic MGD has been observed in *Arabidopsis* mutants which could neither synthesize nor desaturate this prokaryotic chloroplast lipid (Browse *et al.*, 1986a,b, 1989a; Kunst *et al.*, 1988, 1989). But compared with DGD and PG (see below), this increase of 16:0 in MGD is low. This could indicate that the desaturation level of this lipid is kept within narrow limits, whereas in PG and DGD higher levels of 16:0 can be tolerated.

In DGD of both transgenic lines the proportion of 16:0 in *sn*-1 is strikingly increased and higher than the proportion of the prokaryotic species which are characterized by C16 fatty acids at C2. This can be explained by the fact that eukaryotic DGD species always contain significant proportions of 16:0 in *sn*-1, and nearly all 16:0 found in the *sn*-1 position of wild type DGD has to be attributed to eukaryotic species. By substracting this value from that found in transgenic DGD it appears that a high proportion of the prokaryotic DGD is esterified with 16:0 in *sn*-1 as a consequence of the action of the bacterial acyltransferase. Future studies have to show whether 16:0/16:0 species of DGD do in fact accumulate in the transgenic plants, which could also contribute to chilling sensitivity. From studies with spinach chloroplasts it can be inferred that this completely saturated species represents a major proportion of the prokaryotic DGD formed by isolated organelles (Heemskerk *et al.*, 1991). Similar considerations may apply to sulfoquinovosyl diacylglycerol (SQD) which also shows a rise in 16:0.

The most pronounced increase of 16:0 was found in PG which is of particular interest for several reasons. First, PG from chloroplasts is the only lipid class of this organelle which is exclusively synthesized *de novo* within plastids and accordingly has a purely prokaryotic diacylglycerol moiety. Therefore, its fatty acid composition will monitor changes of the biosynthetic activity within these organelles.

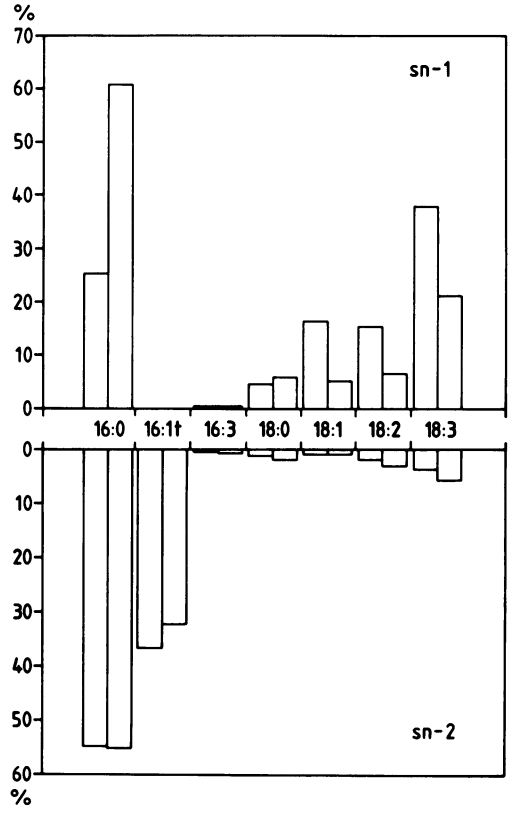


**Fig. 3.** Positional distribution of fatty acids in PG from wild type and transgenic *Arabidopsis thaliana* (line no.4) plants. Fatty acid profiles (mole %) are given for the *sn*-1 and *sn*-2 positions of the glycerol backbone. Fatty acids are characterized by carbon and double bond numbers. 16:1t stands for 3-*trans*-hexadecenoic acid which is confined to the C2 position of chloroplast PG. For each fatty acid the left bar represents the proportion from wild type and the right bar the proportion from transgenic plants.

Furthermore, in leaves most of the PG is localized in plastids as is evident from the low proportion of C18 fatty acids in the *sn*-2 position of leaf PG (Dorne and Heinz, 1989;

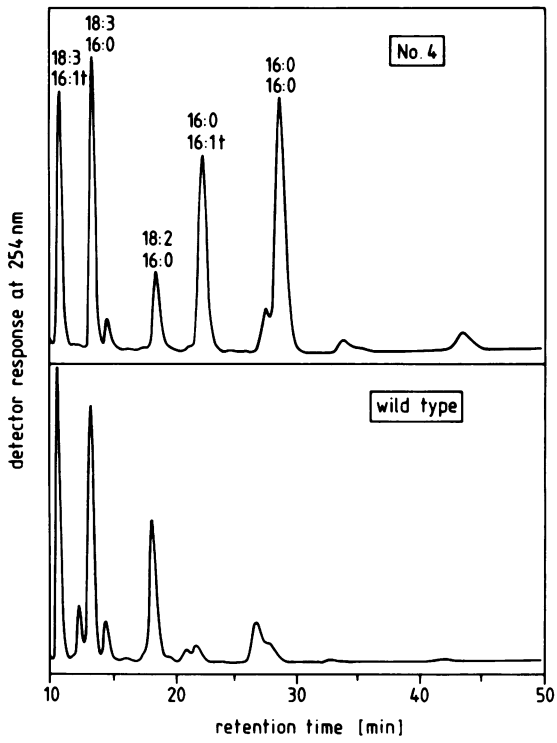


**Fig. 4.** HPLC chromatogram showing the composition of molecular species of PG from wild type and transgenic *Arabidopsis* plants (line no.4). Individual components are identified by their constituent fatty acids with the fatty acid most likely in the *sn*-1 position given above the *sn*-2-bound acyl group.

Table III). Finally, the fatty acid composition of PG and in particular the proportion of non-fluid components as outlined above have been proposed to be one of the factors limiting chilling tolerance.

In PG the 16:0 content was raised from ~35% in wild type to over 50% in the two transgenic lines (Table II). The positional analysis reveals that this increase is confined to the *sn*-1 position, where the proportion of 16:0 is more than doubled. Because of their importance for chilling sensitivity the data of the positional distribution of fatty acids in PG are separately depicted in Figure 3 which in addition show convincingly that the engineering is limited to the *sn*-1 position without any interference at C2.

The fact that the *sn*-2 position of PG is almost completely esterified by 16:0 and *trans*-16:1 (see Table III) in combination with the increase of 16:0 in *sn*-1 of PG in the transgenic lines suggest that a high proportion of hmt-PG with two rigid fatty acids such as 16:0, 18:0 or *trans*-16:1 combined in one lipid molecule should be present in these plants. In Figure 4 the resolution of the molecular species of PG from transgenic and wild type plants is shown. The two hmt species 16:0/*trans*-16:1 and 16:0/16:0 make up <5% of the PG species in the wild type plants, but are increased to >50% in the transformed line.

From the data of our lipid analysis we conclude that in the transgenic plants the correctly translated *PlsB* with its preference for 16:0 displays enzymatic activity leading to an alteration of lipid composition. The fact that the increase is almost limited to plastidial lipids is evidence that most of the enzymatically active *PlsB* is located within the plastids as a consequence of the leader sequence-facilitated import. The activity of *PlsB* redirects available fatty acids with regard to their positional location, and by this the total fatty acid composition is changed to some extent. By combining the data from Tables I and II the overall content of 16:0 can be estimated and an increase of ~50% from 14 mole % in the wild type to 22 mole % in the transgenic lines is calculated. Thus, the bacterial enzyme competes successfully not only with the native GPAT but also with the plastidial elongase and prevents part of the 16:0 from being converted to C18 fatty acids and from being desaturated at C2 of MGD.

### *Stress treatment*

The proportion of hmt-PG in the two transgenic lines exceeds that of many chilling sensitive plants. To test whether this alteration is sufficient to create chilling sensitivity, transgenic as well as wild type plants were incubated at 4°C in the dark. Even after prolonged exposure no significant difference between transgenic and wild type plants was visible. This changed drastically when plants kept for 7 days at 4°C were returned to 20°C. While wild type plants were apparently unaffected by this treatment, the transgenic plants showed wilting in their older leaves which after 2 days became brown and necrotic as shown in Figure 5. Recent data show that incubation of the transgenic plants at low temperature under illumination leads to even more severe symptoms, as plants then started wilting after 3 to 4 days and finally died (data not shown).

The observed increase in chilling sensitivity might be a non-specific decrease in stress tolerance due to a reduced fitness of the transgenic plants. To test this, plants were subjected to a heat stress experiment. Transgenic as well as wild type plants withstand a 2 h treatment at 37°C without visible damage, but neither were able to survive the treatment at 42°C, as described for wild type *Arabidopsis* plants (Binelli and Mascarenhas, 1990). Despite their limitation these results provide first evidence that the increased chilling sensitivity is not the consequence of a general and non-specific loss of stress tolerance, but may specifically be correlated with low temperature performance in dependence of PG fluidity.

## Discussion

We have isolated two independent transgenic *Arabidopsis* lines which express a modified bacterial gene in enzymatically active form. The newly introduced enzymatic activity changes the fatty acid composition of membrane lipids, and those components are most severely affected which are made inside the plastids. This implies that the new activity is targeted to the plastids and thus the N-terminal transit peptide of the rubisco small subunit gene is able to govern the import of this large *PlsB* protein consisting of more than 800 amino acids, which in addition is highly hydrophobic and in its native cell a membrane-bound component.

The transgenic lines show an increase of 16:0 in the *sn*-1 position of nearly all lipids, but this rise is most prominent in those lipids which are synthesized inside the plastid. Whether the slight increase in 16:0 observed in extraplastidic phosphatidylcholine (PC) and phosphatidylethanolamine (PE) is due to residual extraplastidial activity or has to be ascribed to secondary effects such as export from plastids (Browse *et al.*, 1989b) cannot be answered at present. Alternatively, those 16:0 molecules which are released from C1 of MGD

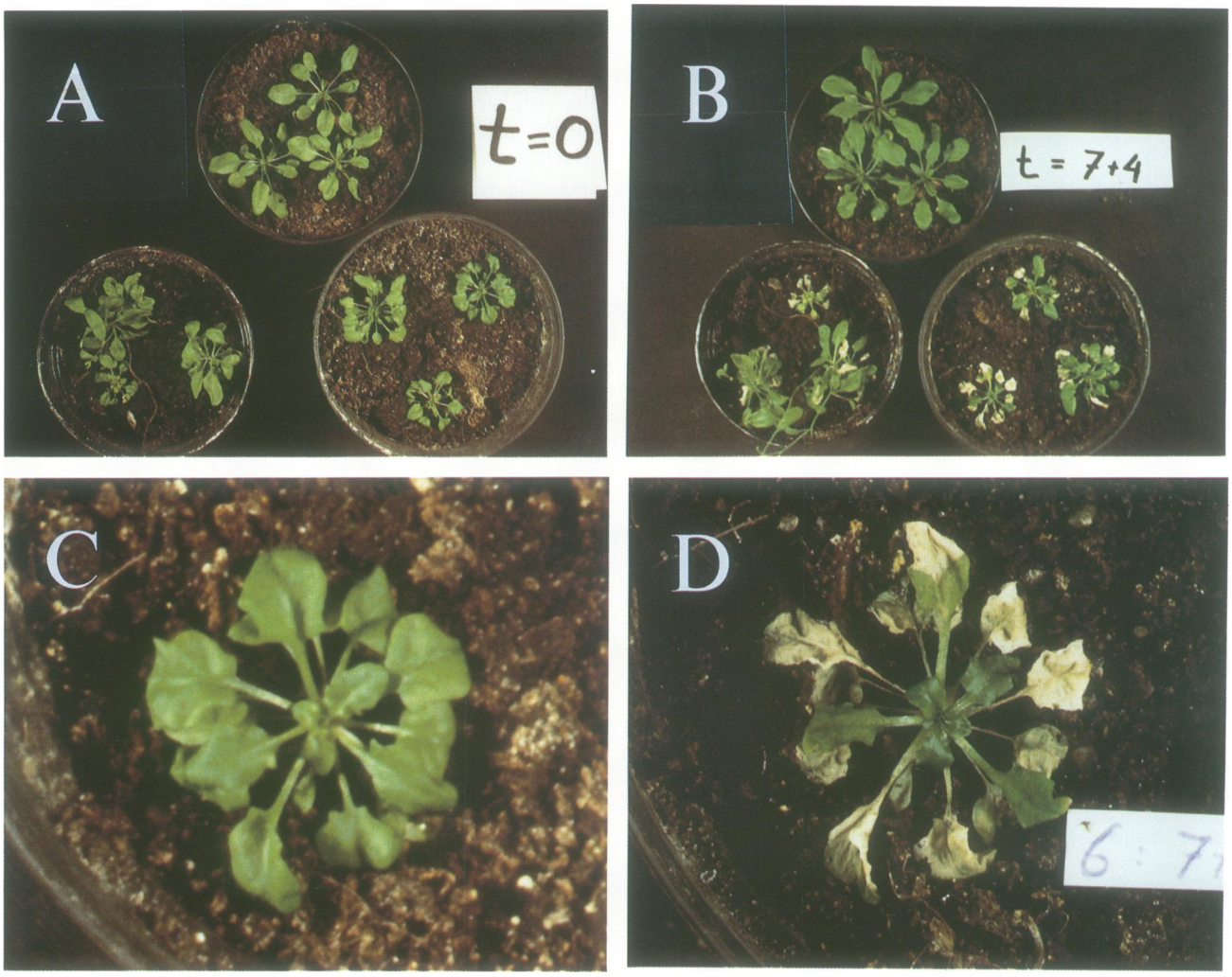


**Fig. 5.** Habitus of wild type and transgenic plants before and after chilling treatment and reculture. Plants are shown before (**A** and **C**) and after (**B** and **D**) they were kept for 7 days at 4°C and an additional 4 days at 20°C. Pots in (A) and (B): top, wild type; bottom left, line no.4; bottom right, line no.6. (C) and (D): the same plant of line no.6 before and after cold treatment in detail.

by enhanced turnover as discussed above may be exported from the plastids into the endoplasmic reticulum and thus lead to a higher incorporation into the *sn*-1 position of extraplastidic lipids.

The high incorporation of 16:0 into *sn*-1 of PG increased the hmt-PG species to such an extent that according to the hypothesis of PG-dependent chilling sensitivity these transgenic plants have to be classified as potentially chilling sensitive. Our *Arabidopsis* plants with the altered lipid composition respond to a cold treatment by developing symptoms of leaf necrosis during recultivation at normal temperature. A retardation in the development of symptoms is often observed in plants with natural chilling sensitivity (Salveit and Morris, 1990), and this delayed response was considered as arguing against the hypothesis of PG-dependent chilling sensitivity, since phase transitions are rapid processes and one would expect an immediate effect on the plant. But the observation of the same delay in damage in our transgenic plants supports the view of a causal correlation between hmt-PG and chilling sensitivity.

On the other hand, our transgenic plants have not only an altered lipid composition, but also a new foreign membrane protein in the plastids. One could argue that the mere presence of this protein might induce the chilling sensitivity and the altered phenotype of our plants. But the data from an independent experiment (Murata *et al.*, 1992) support the conclusion that it is not just the protein, but the consequence of its expressed activity which causes chilling sensitivity in the transgenic plants. In this study tobacco served as target plant and the acyltransferase from squash was used to increase 16:0 in PG. Its expression in transgenic plants led to similar changes in lipid compositions as observed in our studies and in particular, the symptoms of cold treatment became visible during subsequent recultivation under warm conditions and preferentially in older leaves. In contrast to the *E.coli* enzyme in our plants, the squash acyltransferase in tobacco is not membrane bound, but nevertheless the activities of both enzymes result in similar symptoms in two unrelated plant species. This strongly argues against a non-specific effect due to the presence of a foreign protein.

In both studies the increased incorporation of 16:0 is not limited to PG, and as no compensatory increase in desaturation is observed, the overall lipid saturation is increased as well. Therefore, it is not clear whether it is this reduced desaturation or the specifically increased proportion of hmt-PG which leads to chilling sensitivity. In this context recent experiments on the performance of desaturase mutants from *Arabidopsis* at chilling temperature are of particular

relevance (Hughly and Somerville, 1992). In these mutants the level of polyunsaturated fatty acids in plastidic lipids is strikingly reduced, whereas the saturated fatty acids in PG are not affected (Browse *et al.*, 1989a; Kunst *et al.*, 1989) and the proportion of hmt-PG is unchanged. When mutant plants of similar developmental state as used in our experiments were exposed to chilling temperatures, they did not show symptoms of chilling sensitivity. This argues in favour of a specific role of hmt-PG in initiating chilling sensitivity.

Apart from providing supporting evidence for the hypothesis of PG-mediated chilling sensitivity, our experiments have shown that it is possible to alter the composition of plant lipids by genetic engineering at specific points. This opens new access to physiological problems and has relevance for future activities in engineering other aspects of plant lipid metabolism.

## Materials and methods

### Materials

Plasmids: pCV701 (Koncz *et al.*, 1987), pH80 (8.0 kb *Hin*dIII fragment containing rbcS-1 cloned into pUC8) (Wolter *et al.*, 1988), pVL1 (Lightner *et al.*, 1983), pVV1300 (700 bp *Pst*I fragment containing *rbcS-c* in pUC8) (Wolter *et al.*, 1988), pGSC1704 (PGS, Ghent, Belgium).

Plant material: transgenic as well as control plants (*Arabidopsis thaliana*, ecotype Columbia-C24) were kept under permanent light at 20°C. All transgenic plants were germinated under selective conditions in order to ensure hygromycin resistance.

### Plasmid construction

For cloning and transformation of *E.coli*, standard techniques were used (Sambrook *et al.*, 1989). For constructing pHAMPL5 the promoter and coding region for the transit peptide from rbcS-1 were isolated as a 2.5 kilobase pair (kb) *Hin*dII–*Sph*I fragment. The coding region of *plsB* was isolated as a 2.5 kb *Bal*–*Stu*I fragment from pVL1 (Lightner *et al.*, 1983). These fragments were connected by insertion of a short *Sph*–*Bal*I fragment of 9 bp length isolated from rbcS-c, a rubisco cDNA clone (Wolter *et al.*, 1988). This short piece contained the processing site for the transit peptide. The *Stu*I site in pVL1 is located 87 nucleotides downstream from the stop codon of the *plsB* coding region and was fused to the 230 bp *Sal*–*Hin*dIII fragment from pCV701 (Koncz *et al.*, 1987) containing the polyadenylation site of the octopine synthase gene connected by the 15 bp *Sma*–*Sal*I linker from the pUC18 multiple cloning site.

### Raising antibodies against E.coli acyltransferase

To isolate an enriched *PlsB* protein fraction the *Eco*RI insert of pVL1 was transferred into pUC8. The increase in copy number leads to overexpression of the *plsB* gene (not shown). *PlsB* was partially purified following a published protocol (Green *et al.*, 1981). *Escherichia coli* cells harbouring the plasmid were grown overnight at 37°C under vigorous shaking in rich medium. All following steps were carried out at 0–4°C. After pelletting and washing in buffer A (50 mM Tris–HCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, pH 8.5) the cells were broken by ultrasonic treatment. Debris was removed by low speed centrifugation and the supernatant subjected to ultracentrifugation at 200 000 *g* for 60 min. The resulting pellet was resuspended in buffer B [25 mM Tris–HCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 20% (w/v) glycerol, pH 8.5] and after adjusting Triton X-100 to 0.5% (w/v) stirred for ~30 min. After centrifugation at 200 000 *g* for 1 h the supernatant was collected and frozen at −70°C. Preparative SDS gel electrophoresis, immunization of rabbits and preparation of antiserum were carried out as described elsewhere (Weber *et al.*, 1991).

### RNA isolation and Northern blotting

Total RNA was prepared by a modification of the GTC procedure (Chirgwin *et al.*, 1979). For the detection of specific RNA molecules the DIG system (Boehringer, Mannheim) was used which needs labelled RNA as probes. For labelling, the 1.2 kb internal *Pvu*II fragment from *plsB* (bp 1429–2644 in pVL1) was cloned into the *Sma*I site of pBluescriptSK− (pATEC1200) and after linearization 1 μg DNA was used as template to transcribe a digoxigenin-labelled RNA using T7 polymerase.

About 15 μg of total RNA from transgenic and control plants were separated on a 2% agarose gel at 50 V for ~4.5 h. Electrophoresis was carried out according to standard conditions with minor modifications. The RNA was blotted on to a nylon membrane and fixed by UV treatment. Prehybridization, hybridization, washing and detection of the fluorescence signal followed manufacturer's instructions with some modifications (Düring, 1991).

### Isolation of membrane fractions and Western blotting

About 500 mg of fresh leaf material was frozen in liquid nitrogen and ground in a mortar to a fine powder. All following steps were carried out at 0–4°C. The powder was transferred to a small blender and after addition of 5 ml buffer A mixed in intervals for 12 s. After filtration through cheesecloth the membranes were pelleted for 10 min at 3000 *g*. The pellet was resuspended in 500 μl buffer B. Relative sample concentrations were based on chlorophyll content. Aliquots of resuspended membranes equivalent to 60 μg chlorophyll were used to measure enzymatic activity under standard conditions for 20 min at 24°C (Bertrams and Heinz, 1981). Proteins from membrane fractions were separated by standard SDS–PAGE (4.5% stacking gel, 10% separation gel; 30:0.8 acrylamide/bisacrylamide) for 1 h at 200 V. The proteins were transferred to a nylon membrane as described earlier (Weber *et al.*, 1991) with minor variations (1 mA/$cm^2$; transfer buffers contained 0.1% SDS to achieve mobilization of the large hydrophobic *PlsB* polypeptide). For detection of the *PlsB* protein the anti-*PlsB* antibody (dilution 1:500) was employed in the non-radioactive DIG system (Boehringer, Mannheim) with some modifications (Düring, 1991).

### Analysis of lipids and fatty acids

Before lipid extraction, the plant material was placed into boiling water for some minutes. Separation of lipid classes by thin layer chromatography (TLC), quantification of lipids by colorimetry and analysis of fatty acids as bromophenacyl esters have been described before (Haschke *et al.*, 1990). For a positional analysis, leaf lipids extracted from 2–3 g fresh weight were separated by TLC on two Kieselgel 60 plates in chloroform/methanol/acetic acid/water 91:30:4:4 (v/v). The individual compounds were recovered from the appropriate zone and subjected to enzymatic hydrolysis (*Rhizopus* lipase) followed by HPLC of fatty acids as *p*-bromophenacyl esters. Our analysis of wild type PG concurs with the previously determined pattern (Browse *et al.*, 1986a). For the analysis of molecular species from PG, diacylglycerol portions from PG (isolated from 2–3 g of fresh weight) were released enzymatically (phospholipase C) and converted to dinitrobenzoyl derivatives (Takamura *et al.*, 1986) for subsequent reverse-phase HPLC with isocratic elution (acetonitrile/isopropanol, 98:2, v/v, 3 μm RP18 ODS hypersil column, 0.46×12.5 cm). Identification was carried out by using reference species and by collecting individual peaks followed by analysis of constituent fatty acids.

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## References

Bertrams,M. and Heinz,E. (1981) *Plant Physiol.*, **68**, 653–657.
Binelli,G. and Mascarenhas,J.P. (1990) *Dev. Genet.*, **11**, 294–298.
Bishop,D.G. (1986) *Plant Cell Env.*, **9**, 613–616.
Bishop,D.G. and Kenrick,J.R. (1987) *Phytochemistry*, **26**, 3065–3067.
Browse,J., Warwick,N., Somerville,C.R. and Slack,C.R. (1986a) *Biochem. J.*, **235**, 25–31.
Browse,J., McCourt,P. and Somerville,C. (1986b) *Plant Physiol.*, **81**, 859–864.
Browse,J., Kunst,L., Anderson,S., Hugly,S. and Somerville,C. (1989a) *Plant Physiol.*, **90**, 522–529.
Browse,J., Kunst,L., Hugly,S. and Somerville,C. (1989b) In Biacs,P.A., Gruz,K. and Kremmer,T. (eds), *Biological Role of Plant Lipids*. Akadémiai Kladó, Budapest and Plenum Publishing Corporation, New York, pp. 335–339
Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) *Biochemistry*, 18, 5294–5299.
Cronan,J.E.,Jr and Roughan,P.G. (1987) *Plant Physiol.*, **83**, 676–680.
Dorne,A.-J. and Heinz,E. (1989) *Plant Sci.*, **60**, 39–46.
Düring,K. (1991) *Anal. Biochem.*, **196**, 433–438.
Frentzen,M., Heinz,E., McKeon,T.A. and Stumpf,P.K. (1983) *Eur. J. Biochem.*, **129**, 629–636.

Frentzen,M., Nishida,I. and Murata,N. (1987) *Plant Cell Physiol.*, **28**, 1195–1201.
Green,P.R., Merrill,A.H. and Bell,R.M. (1981) *J. Biol. Chem.*, **256**, 11151–11159.
Haschke,H.D., Kaiser,G., Martinoia,E., Hammer,U., Teucher,T., Dorne,A.J. and Heinz,E. (1990) *Botanica Acta*, **103**, 32–38.
Heemskerk,J.W.M., Schmidt,H., Hammer,U. and Heinz,E. (1990) *Plant Physiol.*, **96**, 144–152.
Hughly,S. and Somerville,C. (1992) *Plant Physiol.*, **99**, 197–202.
Koncz,C., Olsson,O., Langridge,W.H.R., Schell,J. and Szalay,A.A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 131–135.
Kunst,L., Browse,J. and Somerville,C. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4143–4147.
Kunst,L., Browse,J. and Somerville,C. (1989) *Plant Physiol.*, **90**, 943–947.
Lightner,V.A., Bell,R.M. and Modrich,P. (1983) *J. Biol. Chem.*, **258**, 10856–10861.
Long,S.P. and Woodward,F.I. (eds) (1988) *Plant and Temperature.* Society for Experimental Biology, Cambridge, UK.
Lyons,J.M. (1973) *Annu. Rev. Plant Physiol.*, **24**, 445–466.
Mudd,J.B., Andrews,J.E. and Sparace,S.A. (1987) *Methods Enzymol.*, **148**, 338–345.
Murata,N. (1983) *Plant Cell Physiol.*, **24**, 81–86.
Murata,N. and Yamaya,J. (1984) *Plant Physiol.*, **74**, 1016–1024.
Murata,N., Ishizaki-Nishizawa,O., Higashi,S., Hayashi,H., Tasaka,Y. and Nishida,I. (1992) *Nature*, **236**, 710–713.
Norman,H.A. and St John,J.B. (1986) *Plant Physiol.*, **81**, 731–736.
Raison,J.K. and Wright,L.C. (1983) *Biochim. Biophys. Acta*, **731**, 69–78.
Rock,C.O., Goelz,S.E. and Cronan,J.E.,Jr (1981) *J. Biol. Chem.*, **256**, 736–742.
Roughan,P.G. (1985) *Plant Physiol.*, **77**, 740–746
Roughan,P.G., Thompson,G.A. and Cho,S.H. (1987) *Arch. Biochem. Biophys.*, **259**, 481–496.
Salveit,M.E.,Jr and Morris,L.L. (1990) In Wang,C.Y. (ed.), *Chilling Injury of Horticultural Crops*. CRC Press, Boca Raton, FL, pp. 3–16.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schmidt,R. and Willmitzer,L. (1988) *Plant Cell Rep.*, **7**, 583–586.
Takamura,H., Narita,H., Urade,R. and Kito,M. (1986) *Lipids*, **21**, 356–361
Weber,S., Wolter,F.P., Buck,F., Frentzen,M. and Heinz,E. (1991) *Plant Mol. Biol.*, **17**, 1067–1076.
Wolter,F.P., Fritz,C.C., Willmitzer,L., Schell,J. and Schreier,P.H. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 846–850.

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