Plant Physiol. (1994) 105: 601–605

Genetic Enhancement of Cold Tolerance by Expression of a Gene for Chloroplast ω-3 Fatty Acid Desaturase in Transgenic Tobacco[1]

Hiroaki Kodama*, Tatsurou Hamada, Gorou Horiguchi, Mitsuo Nishimura, and Koh Iba
Department of Biology, Faculty of Science, Kyushu University 33, Fukuoka 812, Japan

The increased production of trienoic fatty acids, hexadecatrienoic (16:3) and linolenic (18:3) acids, is a response connected with cold acclimation of higher plants and is thought to protect plant cells against cold damage. Transgenic tobacco (*Nicotiana tabacum* cv SR1) plants that contain increased levels of 16:3 and 18:3 fatty acids, and correspondingly decreased levels of their precursors, hexadecadienoic and linoleic acids, were engineered by introduction of a chloroplast ω-3 fatty acid desaturase gene (the *fad7* gene) isolated from *Arabidopsis thaliana*. When exposed to 1°C for 7 d and then cultured at 25°C, the suppression of leaf growth observed in the wild-type plants was significantly alleviated in the transgenic plants with the *fad7* gene. The low-temperature-induced chlorosis was also much reduced in the plants transformed with the *fad7* gene. These results indicate that increased levels of trienoic fatty acids in genetically engineered plants enhance cold tolerance.

Low temperature is one of the major environmental factors that limit plant growth. Many plants of tropical origin suffer cold damage when exposed to temperatures below 20°C (Graham and Patterson, 1982). An increased production of highly unsaturated fatty acids such as 18:3 at low temperature is observed in connection with cold acclimation in many plants (Graham and Patterson, 1982). The crucial role of polyunsaturated fatty acids in cold tolerance has been pointed out in studies with an inhibitor of 18:3 synthesis (St. John et al., 1979) and also with the *fad* mutants of *Arabidopsis thaliana*, which are defective in the desaturation of membrane lipids (Browse and Somerville, 1991; Somerville and Browse, 1991). The *fad* mutants *fad2* (Lemieux et al., 1990), *fad5* (Kunst et al., 1989b), and *fad6* (Browse et al., 1989) have reduced amounts of polyunsaturated fatty acids and are more susceptible to chilling (Hugly and Somerville, 1992; Miquel et al., 1993). Furthermore, cold tolerance was enhanced in cyanobacteria by an increase in the amount of unsaturated fatty acids in the genetically engineered membrane lipids (Wada et al., 1990).

A correlation between chilling sensitivity and the degree of unsaturation of fatty acids in PG in plastid membranes has been reported in higher plants (Murata et al., 1982; Murata, 1983; Tasaka et al., 1990). In this respect, glycerol-3-P acyltransferase is thought to partly determine the level of unsaturated PG. An alteration of chilling sensitivity has been reported in the transgenic tobacco (*Nicotiana tabacum*) (Murata et al., 1992) and *A. thaliana* (Wolter et al., 1992) into which the glycerol-3-P acyltransferase gene was introduced.

The *fad7* mutant of *A. thaliana* is characterized by decreased accumulation of trienoic fatty acids (16:3 and 18:3) and a corresponding increase in the amount of dienoic fatty acids (16:2 and 18:2) in leaf cells (Browse et al., 1986). The effect of the *fad7* mutation shows no specificity in the length of carbon chain (16- or 18-carbon), glycerol backbone (*sn*-1 or *sn*-2), or the lipid head group. Therefore, the formation of trienoic fatty acids of all lipid classes, including PG, is considered to be controlled to a large extent by the *fad7* gene in leaf tissues. Recently, a cDNA encoding the chloroplast ω-3 fatty acid desaturase that complements the *fad7* mutation was isolated (Iba et al., 1993; Yadav et al., 1993). We report here the successful production of transgenic tobacco plants in which the level of trienoic fatty acids is considerably increased by introduction of the *fad7* desaturase gene. In the transgenic tobacco, increased amounts of trienoic fatty acids enhance cold tolerance.

MATERIALS AND METHODS

Plasmid Construction

The construction of pTiDES7 was previously described (Iba et al., 1993). This plasmid is a derivative of pBI121 and contains a chloroplast ω-3 desaturase gene (the *fad7* gene) under the control of the cauliflower mosaic virus 35S promoter. The p501–17 plasmid, a derivative of pBI101, was used as an empty vector. This plasmid lacks the β-glucuronidase gene (*gus* or *uidA*). In both plasmid pTiDES7 and p501–17, the neomycin phosphotransferase II gene was used as a selectable marker.

Plant Transformation

Nicotiana tabacum cv SR1 was transformed by the leaf-disc method (Horsch et al., 1985) by using *Agrobacterium tumefa-*

[1] This research was supported by Grants-in-Aid for Scientific Research (No. 05266214 to K.I. and No. 05854080 to H.K.) from the Ministry of Education, Science and Culture, Japan. This research was also supported in part by the Sumitomo Foundation.

* Corresponding author; fax 81–92–632–2741.

Abbreviations: 16:2, hexadecadienoic acid; 16:3, hexadecatrienoic acid; 18:2, linoleic acid; 18:3, linolenic acid; MS, Murashige-Skoog; PG, phosphatidylglycerol; *RGR*, relative growth rate. ω-3 refers to the position of the double bond from the methyl end of a fatty acid.

ciens C58ClRif (pGV2280) containing the plasmid pTiDES7 or p501–17. Individual kanamycin-resistant regenerated shoots were selected, and the plants were rooted in MS medium with no growth regulators (Murashige and Skoog, 1962) and transferred to soil. Primary transformants (R_0) were selfed by bagging their inflorescences. The R_1 seeds resulting from the self-pollination were aseptically germinated in continuous light (2000 lux) at 25°C on MS medium supplemented with 100 mg/L kanamycin. The kanamycin-resistant R_1 seedlings were subjected to further analyses of lipids and cold tolerance.

Fatty Acid Analysis

Fatty acid methyl esters of leaf samples were prepared as described by Lemieux et al. (1990). Using a capillary column (HR-SS-10, 0.25 mm × 25 m, Shinwa Chemical Industries, Ltd., Kyoto, Japan), the GC (GC-14B, Shimadzu, Kyoto, Japan) was performed under a programmed temperature control (initial temperature 170°C for 3 min followed by an increase of 3°C/min to 210°C and then 5°C/min to 220°C). Fatty acid methyl esters were identified by comparison with the retention times of authentic samples.

Plant Growth Conditions and Cold Treatment

The wild-type and transgenic seeds were germinated at 25°C on MS agar medium with or without 100 mg/L kanamycin. The kanamycin-resistant R_1 seedlings and the wild-type seedlings were grown on MS agar medium in Petri dishes at 25°C under continuous illumination by white fluorescent lamps at an intensity of 2000 lux. For the cold treatment, the following procedures were carried out. When very small first and second leaves emerged (usually about 6 d after germination at 25°C), plants in Petri dishes were exposed to 1°C for 7 d in a low-temperature incubator (LTI-1000SD, Tokyo Rikakikai, Tokyo, Japan) in continuous light (2000 lux) and returned to the original growth conditions. During the cold treatment, plants were arranged randomly in the chamber to avoid local variations in the environment.

Growth Measurement

Areas of the first and second leaves of the kanamycin-resistant R_1 plants and the wild-type plants were measured. The leaf area was approximated by an ellipse. *RGR* is expressed as follows:

$$RGR = (\ln A_t - \ln A_0)/t$$

where A_0 and A_t are the initial and the final leaf areas and t (d) is the duration of incubation.

RNA Blot Analysis

Total RNA was purified from leaf tissue as described (Ausubel et al., 1987). Twelve micrograms of total RNA were denatured and fractionated as described previously (Kodama et al., 1991). The 3′ noncoding sequence of the *fad7* cDNA clone (Iba et al., 1993) and the rice tRNA-Gly(GCC) gene (Reddy and Padayatty, 1988) were used as probes. We confirmed that the rice tRNA-Gly(GCC) gene was hybridized to the corresponding tobacco RNA and used it as a reference in the estimation of the amount of loaded RNA. RNAs were transferred to nylon membranes and hybridized with labeled probes. Prehybridization, hybridization, and posthybridization steps were performed essentially as described in the instructions for the nylon membranes (Pall Ultrafine Filtration Corp., New York, NY).

RESULTS

Fatty Acid Composition in Leaves of Transgenic Plants

The *fad7* gene was introduced into tobacco plants under transcriptional control of cauliflower mosaic virus 35S promoter by *Agrobacterium*-mediated transformation. A line of the tobacco transformed with the empty vector p501–17 is designated EV. Of the six independent transformants with the *fad7* gene, three lines (designated SRT-1, SRT-5, and SRT-6) contained substantially increased amounts of both 18:3 and 16:3 and correspondingly decreased amounts of both 18:2 and 16:2 compared with the wild-type and EV plants (Table I). The total content of trienoic fatty acids increased by about 10% in leaves of the SRT-1, -5, and -6 plants. These three transgenic lines segregated as single functional T-DNA insertion plants (data not shown). In this report, these three lines, SRT-1, -5, and -6, are generically named SRT plants.

The *fad7* mRNA was detected in total RNAs prepared from

Table I. *Fatty acid composition of whole leaves of wild-type (WT), EV, and SRT plants grown at 25°C*

The kanamycin-resistant R_1 seedlings and the wild-type seedlings grown on MS medium were transferred to soil and cultured in continuous light (2000 lux) at 25°C. The fatty acid composition was determined in the 1-cm-long third leaves. The values are mol% ± SD (n = 3 or 4).

Fatty Acid	WT	EV	SRT-1	SRT-5	SRT-6
16:0	12.0 ± 0.3	12.7 ± 0.4	10.4 ± 0.6	11.3 ± 0.5	10.1 ± 0.2
16:1	2.1 ± 0.2	2.9 ± 0.2	2.1 ± 0.2	1.9 ± 0.3	3.0 ± 0.2
16:2	1.5 ± 0.4	1.8 ± 0.2	0.2 ± 0.2	Trace	0.4 ± 0.3
16:3	7.9 ± 0.9	7.5 ± 0.3	11.3 ± 0.2	8.7 ± 1.1	13.2 ± 1.5
18:0	1.4 ± 0.3	1.3 ± 0.1	0.9 ± 0.1	0.4 ± 0.6	1.0 ± 0.3
18:1	2.3 ± 0.2	2.5 ± 0.1	1.6 ± 0.1	3.3 ± 0.3	1.9 ± 0.3
18:2	19.5 ± 0.6	20.1 ± 0.8	14.4 ± 0.2	15.7 ± 0.7	14.2 ± 0.5
18:3	53.3 ± 0.8	51.3 ± 0.9	59.2 ± 0.7	58.8 ± 0.2	56.6 ± 0.9
16:3 + 18:3	61.2 ± 0.5	58.8 ± 1.1	70.5 ± 0.6	67.5 ± 1.0	69.8 ± 1.8

the leaves of SRT plants, whereas no hybridization band was detected in total RNA isolated from the wild-type leaves (Fig. 1). Therefore, the high-trienoic-fatty-acid phenotype was associated with the expression of the *fad7* gene of pTiDES7.

Cold Tolerance of Wild-Type and Transgenic Plants with the *fad7* Gene

We investigated the effects of the increased amounts of trienoic fatty acids on cold tolerance. The light-grown (2000 lux, 25°C) SRT, EV, and wild-type seedlings, which had a pair of very small leaves (the first and second leaves), were grown further under continuous light (2000 lux) at 15°C and 25°C for 5 d. Then the *RGR* was calculated for the first and second leaves from the increase in the leaf area. The *RGR*s of the second leaf of these plant lines grown at 15°C were reduced to about 50 to 70% of those grown at 25°C, but there were no significant differences among the plant lines (Fig. 2Aa). This indicates that in the short term all plant lines tested grew similarly under the moderately low temperature (15°C) condition. On the contrary, when the seedlings grown at 25°C were exposed to 1°C for 7 d at the same growth stage and cultured further at 25°C for 5 d, the *RGR*s determined for the second leaf showed larger differences among the plant lines. The *RGR*s of the wild-type and EV plants after the cold treatment were reduced to 40 to 60% of those of the wild-type and EV plants grown at 25°C, but the *RGR*s of the cold-treated SRT plants were more than 90% of those of the SRT plants grown at 25°C (Fig. 2Ab). Statistical tests showed a significant difference between the control plants (the wild-type and EV plants) and the SRT plants in *RGR* of cold-treated leaves ($P < 0.01$). These results indicated that the low-temperature-induced suppression in *RGR* of the wild-type and EV plants was significantly alleviated in the SRT plants. This alleviation of the chilling-induced inhibition of leaf growth was evident also in the first leaves of the SRT plants (data not shown). In fact, the leaf areas of the second leaves of the cold-treated SRT plants were significantly larger than those of cold-treated wild-type plants (Fig. 2B). At d 8 after the cold treatment, average areas of the first and second leaves of the wild-type plants were 63 and 42% of those of the SRT plants, respectively.

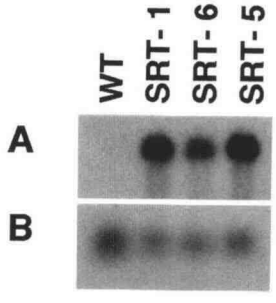

Figure 1. Presence of the *fad7* mRNA in leaves of the wild-type (WT) and three SRT lines. A, Blot probed with 3′ flanking sequence of the *fad7* cDNA. B, Blot reprobed with a gene for rice tRNA-Gly(GCC) to verify that approximately the same amount of total RNA was loaded per lane.

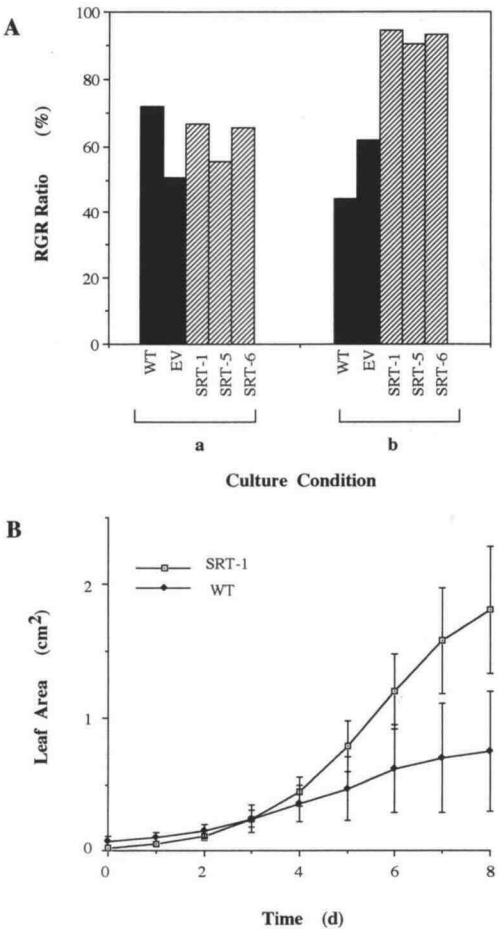

Figure 2. Growth characteristics of the wild-type (WT), EV, and SRT plants under various temperature conditions. A, The *RGR*s of the second leaf of the WT, EV, and SRT plants grown at 15°C for 5 d (a) or at 25°C for 5 d after the exposure to low temperature (1°C) for 7 d (b). *RGR*s are expressed as the ratios to the *RGR* of the control plants grown at 25°C for 5 d at the same growth stage. The sample size was 10 in all cases. Similar results were obtained in three additional, independent experiments. B, Growth of the second leaf of the wild-type ($n = 6$) and the transgenic ($n = 8$) plants (SRT-1). Seedlings were exposed to low temperature (1°C) for 7 d and transferred to a normal culture temperature (25°C). Leaf area was determined at daily intervals after the transfer to the normal temperature. Error bars indicate SD values.

The levels of trienoic fatty acids in the leaf and cotyledon tissues stayed constant during the cold treatment (Table II). Therefore, the effect of the cold treatment (1°C for 7 d) on the composition of fatty acids was insignificant. No visible damage was observed in all tested plants during the cold treatment. Symptoms of leaf chlorosis became obvious in some plants 2 d after the transfer to 25°C. Compared with the SRT plants, the wild-type and EV plants were more susceptible to low-temperature treatment and liable to leaf chlorosis (Table III).

DISCUSSION

Polyunsaturation of fatty acids is considered to be a key factor in the adaptation to low temperatures in higher plants.

Although the *fad7* mutation was deficient in the desaturation of dienoic fatty acids in leaf lipids, the effects on the level of lipid unsaturation of the *fad7* mutation were much less pronounced in plants grown at low temperature (Browse et al., 1986). An isozyme of the *fad7* desaturase, which is induced at low temperature in *A. thaliana*, is assumed to compensate for the deficiency (Somerville and Browse, 1991). The *desA* gene, which encodes Δ12 desaturase of *Synechocystis* PCC6803, also showed a low-temperature-induced expression (Los et al., 1993). The enhancement of the ability to increase the amounts of polyunsaturated fatty acids at low temperature may indicate the importance of lipid polyunsaturation in the maintenance of biological systems under chilling conditions.

The SRT plants showed that increased amounts of trienoic fatty acids could alleviate the cold damage. This alleviation was observed in two low-temperature-induced symptoms, leaf chlorosis and suppression of leaf growth. This cold-induced damage appeared on very young leaves. The growth rates of the first and second leaves of the cold-treated SRT plants were substantially higher than those of the cold-treated wild-type and EV plants, whereas in all the plants tested the growth characteristics at 15°C and 25°C were similar in the short term. During culture at 25°C after the cold treatment, the third and fourth leaves appeared. The growth rates of these newly emerged leaves were approximately the same in the SRT, EV, and wild-type plants (data not shown). These observations indicate that the effects of increased amounts of trienoic fatty acids on the growth characteristics of leaves were more significant in the developing leaves exposed to low temperature.

Two independent mutants of *A. thaliana*, *fad5* and *fad6*, are deficient in chloroplast ω-9 desaturase (Kunst et al., 1989b) and chloroplast ω-6 desaturase (Browse et al., 1989), respectively. The comparison of the growth and development of the *fad5* and *fad6* mutants with that of the wild-type plants revealed that the polyunsaturated lipids of the chloroplast membrane are required for chloroplast biogenesis at low temperature (Hugly and Somerville, 1992). Both of the mutants contained markedly reduced amounts of trienoic fatty acids, especially 16:3. The effect of low temperature on leaf chlorosis of the *fad5* and *fad6* mutants was more pronounced in young leaves than in old leaves. These observations on the *fad* mutants are consistent with the findings obtained from the present analyses of growth and development of the SRT plants. Taken together, the results obtained here indicate that the trienoic fatty acids are undoubtedly an important factor contributing to cold tolerance.

Table II. *Levels of trienoic fatty acids in leaf and cotyledon tissues before and after the cold treatment at 1°C for 7 d under continuous illumination (2000 lux)*

The values are mol% ± SD (n = 3 or 4).

Plant Line	Amount of Trienoic Fatty Acids	
	1°C, 0 d	1°C, 7 d
	mol %	
EV	51.8 ± 3.0	52.7 ± 2.2
SRT-1	58.7 ± 1.9	58.8 ± 1.2

Table III. *Visual assessment of leaf chlorosis in tobacco plants*

Wild-type (WT), EV, and SRT plants were exposed to 1°C for 7 d and then transferred to 25°C for 3 d.

Plant Line	Plants with Leaf Chlorosis	Total Number of Plants (n)
Experiment 1		
WT	5	9
SRT-1	0	9
Experiment 2		
WT	7	10
EV	5	10
SRT-1	1	10
SRT-5	1	10
SRT-6	0	10

Introduction of genes for glycerol-3-P acyltransferase to higher plants has been reported (Murata et al., 1992; Wolter et al., 1992). However, the increase in amounts of polyunsaturated fatty acids was slight in these transgenic plants. In fact, an *A. thaliana* mutant, *act1* (Kunst et al., 1989a), which is deficient in glycerol-3-P acyltransferase, showed less cold-induced chlorosis than the *fad5* and *fad6* mutations. The *fad7* gene and the *fad3* gene, which is a recently isolated gene for the microsomal ω-3 desaturase (Arondel et al., 1992; Yadav et al., 1993), are appropriate tools for the increased production of trienoic fatty acids. The availability of these genes should provide a novel method of production of transgenic plants with enhanced cold tolerance.

ACKNOWLEDGMENTS

The authors wish to thank Prof. H. Kamada, University of Tsukuba, and Dr. M.T. Ueguchi, Nagoya University BioScience Center, for kindly supplying us the wild-type tobacco seeds and seeds of the EV plant, respectively.

Received December 28, 1993; accepted March 3, 1994.

LITERATURE CITED

Arondel V, Lemieux B, Hwang I, Gibson S, Goodman HM, Somerville C (1992) Map-based cloning of a gene controlling omega-3 fatty acid desaturation in *Arabidopsis*. Science **258:** 1353–1355

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, eds (1987) Current Protocols in Molecular Biology. Wiley Interscience, New York

Browse J, Kunst L, Anderson S, Hugly S, Somerville C (1989) A mutant of *Arabidopsis* deficient in the chloroplast 16:1/18:1 desaturase. Plant Physiol **90:** 522–529

Browse J, McCourt P, Somerville C (1986) A mutant of *Arabidopsis* deficient in $C_{18:3}$ and $C_{16:3}$ leaf lipids. Plant Physiol **81:** 859–864

Browse J, Somerville C (1991) Glycerolipid synthesis: biochemistry and regulation. Annu Rev Plant Physiol Plant Mol Biol **42:** 467–506

Graham D, Patterson BD (1982) Responses of plants to low, non-freezing temperatures: proteins, metabolism, and acclimation. Annu Rev Plant Physiol **33:** 347–372

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science **227:** 1229–1231

Hugly S, Somerville C (1992) A role for membrane lipid polyunsaturation in chloroplast biogenesis at low temperature. Plant Physiol **99:** 197–202

Iba K, Gibson S, Nishiuchi T, Fuse T, Nishimura M, Arondel V, Hugly S, Somerville C (1993) A gene encoding a chloroplast omega-3 fatty acid desaturase complements alterations in fatty acid desaturation and chloroplast copy number of the *fad7* mutant of *Arabidopsis thaliana*. J Biol Chem **268:** 24099–24105

Kodama H, Ito M, Ohnishi N, Suzuka I, Komamine A (1991) Molecular cloning of the gene for plant proliferating-cell nuclear antigen and expression of this gene during the cell cycle in synchronous cultures of *Catharanthus roseus* cells. Eur J Biochem **197:** 495–503

Kunst L, Browse J, Somerville C (1989a) Altered chloroplast structure and function in a mutant of *Arabidopsis* deficient in plastid glycerol-3-phosphate acyltransferase activity. Plant Physiol **90:** 846–853

Kunst L, Browse J, Somerville C (1989b) A mutant of *Arabidopsis* deficient in desaturation of palmitic acid in leaf lipids. Plant Physiol **90:** 943–947

Lemieux B, Miquel M, Somerville C, Browse J (1990) Mutants of *Arabidopsis* with alterations in seed lipid fatty acid composition. Theor Appl Genet **80:** 234–240

Los D, Horvath I, Vigh L, Murata N (1993) The temperature-dependent expression of the desaturase gene *desA* in *Synechocystis* PCC6803. FEBS Lett **318:** 57–60

Miquel M, James D, Dooner H, Browse J (1993) *Arabidopsis* requires polyunsaturated lipids for low-temperature survival. Proc Natl Acad Sci USA **90:** 6208–6212

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497

Murata N (1983) Molecular species composition of phosphatidylglycerols from chilling-sensitive and chilling-resistant plants. Plant Cell Physiol **24:** 81–86

Murata N, Ishizaki-Nishizawa O, Higashi S, Hayashi H, Tasaka Y, Nishida I (1992) Genetically engineered alteration in the chilling sensitivity of plants. Nature **356:** 710–713

Murata N, Sato N, Takahashi N, Hamazaki Y (1982) Compositions and positional distributions of fatty acids in phospholipids from leaves of chilling-sensitive and chilling-resistant plants. Plant Cell Physiol **23:** 1071–1079

Reddy PS, Padayatty JD (1988) Effects of 5′ flanking sequences and changes in the 5′ internal control region on the transcription of rice $tRNA^{Gly}$ (GCC) gene. Plant Mol Biol **11:** 575–583

Somerville C, Browse J (1991) Plant lipids: metabolism, mutants, and membranes. Science **252:** 80–87

St. John JB, Christiansen MN, Ashworth EN, Gentner WA (1979) Effect of BASF 13–338, a substituted pyridazinone, on linolenic acid levels and winterhardiness of cereals. Crop Sci **19:** 65–69

Tasaka Y, Nishida I, Higashi S, Beppu T, Murata N (1990) Fatty acid composition of phosphatidylglycerols in relation to chilling sensitivity of woody plants. Plant Cell Physiol **31:** 545–550

Wada H, Gombos Z, Murata N (1990) Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation. Nature **347:** 200–203

Wolter FP, Schmidt R, Heinz E (1992) Chilling sensitivity of *Arabidopsis thaliana* with genetically engineered membrane lipids. EMBO J **11:** 4685–4692

Yadav NS, Wierzbicki A, Aegerter M, Caster CS, Pérez-Grau L, Kinney AJ, Hitz WD, Booth JR, Schweiger B, Stecca KL, Allen SM, Blackwell M, Reiter RS, Carlson TJ, Russell SH, Feldmann KA, Pierce J, Browse J (1993) Cloning of higher plant ω-3 fatty acid desaturases. Plant Physiol **103:** 467–476