

ACCLIMATIVE RESPONSE TO TEMPERATURE STRESS IN HIGHER PLANTS: Approaches of Gene Engineering for Temperature Tolerance

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■ **Abstract** Temperature stresses experienced by plants can be classified into three types: those occurring at (a) temperatures below freezing, (b) low temperatures above freezing, and (c) high temperatures. This review outlines how biological substances that are deeply related to these stresses, such as heat-shock proteins, glycinebetaine as a compatible solute, membrane lipids, etc., and also detoxifiers of active oxygen species, contribute to temperature stress tolerance in plants. Also presented here are the uses of genetic engineering techniques to improve the adaptability of plants to temperature stress by altering the levels and composition of these substances in the living organism. Finally, the future prospects for molecular breeding are discussed.

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INTRODUCTION

Because plants lack the capability of locomotion as a means of responding to changes in their environment, they are exposed to various environmental stresses and must adapt to them in other ways. The most typical kind of stress plants receive

from their surroundings is temperature stress. The range of temperatures experienced by plants varies both spatially and temporally at several different scales. For example, inland equatorial regions can experience maximum daytime temperatures as high as 60°C, whereas in Arctic eastern Siberia, temperatures of almost 30°C in the summer can fall to -70°C in severe winters, a range of 100°C. Altitude and local topography can also exert a significant influence. Each plant species has its own optimum temperature for growth, and its distribution is determined to a major extent by the temperature zone in which it can survive. Efforts in plant breeding have been made to efficiently cultivate varieties of plants suitable for food or livestock feed and to extend their natural range of growth and distribution. The long history of these classical genetic approaches has produced substantial successes, such as increasing the low-temperature tolerance of subtropical rice cultivars, enabling it to yield crops even in subarctic regions (87).

Recently, concerns have been voiced about the potentially serious effects on agriculture of radical global temperature change in the near future. By the latter half of the twenty-first century, global warming that results from increasing atmospheric concentrations of carbon dioxide and other greenhouse gasses could jeopardize agriculture, forestry, and other industries utilizing the natural environment (30). However, although much research has been conducted to evaluate the effects of global warming on these industries (10, 69, 81, 82, 93), efforts to search for specific and practical approaches to improve the adaptability of plants to their temperature environment have only recently begun (19, 91). In recent years, developments in the field of molecular biology have focused attention on molecular breeding methods in addition to those used in classical plant breeding. Rapid advances in molecular genetic approaches have enabled genes to be cloned, both from prokaryotes and directly from the plants themselves, that are thought to provide the key to the mechanism of temperature adaptation (70, 102). Techniques for efficiently and directly introducing genes to a variety of useful plants are also being developed and improved, and progress is being made on the practical application of such techniques (40, 56, 67). This review provides an overview of the genetic engineering approaches currently being used to improve plant tolerance to low- and high-temperature stresses and discusses future prospects. The major characteristics of the genetically engineered temperature stress tolerant plants produced so far together with specific information about the genetic engineering approaches used are summarized in Table 1.

HEAT-SHOCK PROTEINS

The heat-shock response is a reaction caused by exposure of an organisms tissue or cells to sudden high temperature stress, and it is characterized by a transient expression of heat-shock proteins (HSPs). The primary protein structure for HSPs is well conserved in organisms ranging from bacteria and other prokaryotes to eukaryotes such as higher animals and plants. Consequently, it is thought to be closely involved in the protection of the organism against heat stress and the maintenance of homeostasis (61).

TABLE 1 Genetically engineered alterations in the temperature stress tolerance of plants

Functions of transformed genes	Host plants	Effects on the enhancement of temperature and other stress tolerances	Remarks	References
Heat shock proteins				
Heat shock transcription factor (ATHSF1)	Arabidopsis	High temperatures (enhanced)		44
Heat shock protein (Hsp70)	Arabidopsis	High temperatures (lessened)	Heat-inducible antisense expression	45
Small heat shock protein (Hsp17.7)	Carrot	High temperatures (enhanced/lessened)	Constitutive expression, heat-inducible	49
Active oxygen species				
Chloroplast CuZn-superoxide dismutase	Tobacco	Intense light under low temperatures (enhanced)		88, 89
Mn-superoxide dismutase	Alfalfa	Low or freezing temperatures (enhanced)	Targeted to mitochondria or to chloroplast	54
Fe-superoxide dismutase	Alfalfa	Low or freezing temperatures (enhanced)	Targeted to chloroplast	55
Glutathione reductase	Tomato	Not ascertained	Gene from <i>E. coli</i> , targeted to chloroplast	14
Glutathione S-transferase/ Glutathion peroxidase	Tobacco	Low temperatures and salt (enhanced)		84
Compatible solutes				
Betaine aldehyde dehydrogenase	Rice	High and low temperatures and salt (enhanced)	Targeted to peroxisomes, betaine aldehyde applied exogenously	37
Choline oxidase	Arabidopsis	High, low, and freezing temperatures and salt (enhanced)	Gene from a soil bacterium, targeted to chloroplast	2, 3, 22, 85
Membrane lipids				
Glycerol-3-phosphate acyltransferase	Tobacco	Low temperatures (enhanced/lessened)		60, 64
Glycerol-3-phosphate acyltransferase	Rice	Low temperatures (enhanced)		112
Glycerol-3-phosphate acyltransferase from <i>E. coli</i>	Arabidopsis	Low temperatures (lessened)		106
Chloroplast ω -3 fatty acid desaturase	Tobacco	High and low temperatures (enhanced)	Overexpression (low temperatures), gene silencing (high temperatures)	38, 39, 62
Endoplasmic reticulum ω -3 fatty acid desaturase	Tobacco	Not ascertained		20

(Continued)

TABLE 1 (Continued)

Functions of transformed genes	Host plants	Effects on the enhancement of temperature and other stress tolerances	Remarks	References
Endoplasmic reticulum ω -3 fatty acid desaturase	Rice	Low temperatures (enhanced)		92
D9 desaturase	Tobacco	Low temperatures (enhanced)	Gene from a cyanobacterium	32
Transcriptional factors (activators)				
Transcriptional activator (CBF1)	Arabidopsis	Freezing (enhanced)	Induction of COR gene expression	33
cis-acting promoter element (DREB1A)	Arabidopsis	Freezing, drought, and salt (enhanced)	Driven by the rd29A promoter	34
Zinc finger protein (SCOF-1)	Arabidopsis Tobacco	Low temperatures (enhanced)	Induction of COR gene expression	36
Transcriptional activator (ABI3)	Arabidopsis	Freezing (enhanced)	Enhancement of ABA-induced expression of genes for cold acclimation	100

The induction of HSPs is dependent on the temperature at which each species ordinarily grows. In higher plants, HSPs are generally induced by a short exposure to a temperature of 38–40°C. HSPs exist in various molecular sizes, all of which are characterized by binding to structurally unstable proteins. They perform important physiological functions as molecular chaperones (11). In addition to their functions of folding proteins immediately after translation and the transformation of proteins into a structure suited to membrane transport, they prevent the aggregation of denatured proteins and promote the renaturation of aggregated protein molecules. These functions of HSPs are deeply involved in resistance to temperature and various other kinds of stress (11). HSPs are classified into five classes based on their differences in molecular weight [HSP 100, HSP 90, HSP 70, HSP 60, and low-molecular weight HSPs (smHSP)] and are located in both the cytoplasm and organelles such as the nucleus, mitochondria, chloroplasts, and endoplasmic reticulum (ER).

The smHSPs, with a molecular weight of 15–30 kDa, are the most diverse HSPs (8). The pea HSP 18.1 (a cytosolic class I smHSP) works to prevent the aggregation of proteins denatured by heat and to reactivate them, and it has an ATP-independent molecular chaperone activity, as shown through *in vitro* experimental systems on model proteins such as citrate synthase (43).

Expression of the HSP genes is regulated mainly at the transcription level, and the heat-shock activated transcription factors (HSFs) recognize the cis-elements (heat-shock elements; HSEs) located in the upstream promoter region of the HSP

genes, inducing their transcription (107). When an HSF gene was introduced into *Arabidopsis* to cause constitutive expression of HSP, increased thermotolerance compared to wild-type strains was observed (44). At least one smHSP localized in the cytoplasm was expressed in the transgenic *Arabidopsis*, under both non-stress and stress conditions, suggesting that smHSPs may contribute to the plants thermotolerance.

HSP 70 has the most-conserved primary protein structure across different species. Detailed research on it, termed DnaK in *Escherichia coli*, as well as in animal cells and yeasts, has indicated that it functions as a molecular chaperone. HSP 70 is thought to interrupt the interaction within and between protein molecules, for example, to facilitate their membrane transport, to bind to the ER, or to prevent the aggregation of denatured proteins. These functions are ATP dependent, and there are indications, even in plants, for an intrinsic ATPase activity of HSPs (57). In transgenic *Arabidopsis*, transformed with the heat-shock inducible antisense gene for HSP 70, repression of endogenous heat-induced HSP 70 resulted, and a lowering of thermotolerance was observed in the leaf tissues (45).

HSP 100 and HSP 90 are also suspected to function as chaperones, although there is little direct evidence related to temperature stress in plants. However, studies of *Arabidopsis* (24, 25) have been reported in which a mutant, *hot1*, lacking in HSP 101, showed a susceptibility to high temperatures.

Apart from heat shock, there are also HSPs induced by osmotic and salt stress, stress from low oxygen, dinitrophenol (DNP), arsenic compounds, other chemical agents, and plant hormones such as abscisic acid and ethylene. They might play a specific role in the denaturation of proteins, the prevention of that denaturation, and the repair function, as part of the physiological responses to diverse environmental stresses. It has been reported, for example, that salt and drought tolerance improves when the HSP gene is overexpressed in tobacco (97) and in *Arabidopsis* (98).

ACTIVE OXYGEN SPECIES DETOXIFICATION SYSTEMS

The active oxygen species (AOS) includes the superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) that are generated by the reduction of molecular oxygen (O_2) and the hydroxyl radicals ($OH\bullet$) that are produced in reactions involving H_2O_2 and O_2^- . AOS causes damage to cells by oxidation of their constituents. The amount of AOS in plants increases when they are exposed to low temperatures, drought, high light intensity, and other stresses (9). Six enzymes, namely superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR), are involved in AOS-detoxification systems in higher plants. SOD catalyzes the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen in that detoxification process. Hydrogen peroxide is also detoxified by APX and CAT. The three enzymes MDAR, DHAR, and GR are involved in the recovery of ascorbic acid that has been oxidized by APX. CAT is localized in the microbodies

of almost all plant species, but the other five enzymes are found as multiple isozymes, so that a series of these detoxifying enzymes exists in the chloroplast, cytoplasm, mitochondria, and microbodies, respectively.

In *Arabidopsis* subjected to low temperatures, H_2O_2 accumulates in the cells, and the enzyme activities of APX and GR increase (76). Conversely, in rice, APX and SOD levels rise when plants are transferred from cold to normal temperature (86). When tobacco plants are chilled under normal lighting conditions, chloroplastic Fe-SOD is induced, followed by cytosolic CuZn-SOD induction (103). Conspicuous induction of cytosolic CuZn-SOD also takes place under high-temperature treatment in both dark and light conditions. Cytosolic APX is induced by high temperatures, but the high-temperature induction of the gene (*APX1*) for cytosolic APX in *Arabidopsis* is regulated by the HSE (59, 96). Because enzymes related to such AOS detoxification are induced when plants are subjected to the above stresses, it was originally hoped that, by artificially introducing those genes into plants, their tolerance against temperature-induced stress could be increased. The attempts described below, most of which concentrated on the SOD genes, were carried out on the basis of such expectations. Transgenic tobacco, into which chloroplastic CuZn-SOD has been introduced, shows improved resistance to intense light and low temperatures (88, 89), whereas transgenic alfalfa potently expressing Mn-SOD and Fe-SOD in the mitochondria and chloroplasts showed improved resistance to low temperatures (54, 55). However, with transgenic tomato plants overexpressing the *E. coli* GR gene in order to increase the activation of GR, no improvement in chilling tolerance was observed (14). Glutathione S-transferase (GST) is a detoxifying enzyme that uses H_2O_2 and peroxides as substrate. Transgenic tobacco expressing GST genes in the cytoplasm shows resistance to low temperature and salt stress during the process of germination (84).

AOS-detoxification systems are composed of multiple enzymes, and these are thought to function in accordance with stress conditions in different compartments of the cell. Therefore, the protective system might not be able to function more efficiently simply by introducing each individual enzyme gene. On the basis of findings so far, there appear to be limits to the increase in resistance to stress that can be achieved by the introduction of enzyme genes involved in AOS detoxification. To create potent stress tolerant plants, a new strategy will be required to make the entire AOS-detoxification system work efficiently in a well-balanced manner.

GLYCINEBETAINE

When plants are exposed to salt, drought, and low-temperature stress, they accumulate highly soluble organic compounds of low molecular weight, called compatible solutes. These organic compounds exist in stable form inside cells and are not easily metabolized, but neither do they have any effect on cell functions, even when they have accumulated in high concentrations (111). The functions of these substances within the living organism are still unclear. However, because many plant stresses

cause cell dehydration, the accumulation of these substances might play a part in increasing internal osmotic pressure and preventing loss of water from the cell. Typical compatible solutes include mannitol and other sugar alcohols, amino acids such as proline, and amino acid derivatives such as glycinebetaine. Some of these compatible solutes, like proline, are accumulated in practically all plant species, whereas others, like glycinebetaine, are distributed only among plants with a high tolerance to salt or cold (80).

Glycinebetaine is created from choline. In many plants, it is synthesized through a two-step oxidation of choline, by choline monoxygenase and betaine aldehyde dehydrogenase (4, 15, 109). The two enzyme genes have been cloned from plants (31, 68, 79, 104), and attempts have been made to introduce them separately into plants to generate glycinebetaine, but sufficient quantities of the choline substrate and the intermediate betaine aldehyde could not be obtained within the cell (75). For this and other reasons, generating a sufficient quantity of glycinebetaine *in vivo* to contribute to a significant stress tolerance has not been possible. However, when a transgenic rice plant containing the betaine aldehyde dehydrogenase gene was supplied with sufficient exogenous betaine aldehyde to artificially generate a considerable quantity of glycinebetaine, it showed improved tolerance against both salt and low temperatures (37).

In a different pathway, *E. coli* and *Arthrobacter globiformis* are both able to synthesize glycinebetaine from choline, using choline dehydrogenase and choline oxidase, respectively. When the bacterial choline oxydase gene (*CodA*) is introduced into tobacco or *Arabidopsis*, glycinebetaine is synthesized as a result, and not only is salt tolerance increased, but a slight though significant resistance to low temperature and freezing stress is also conferred (2, 22, 85). Equally, the lowered germination rate of seeds under high temperatures is reversed to some extent (3). However, the accumulation is minute ($1 \mu\text{mol GlyBet g}^{-1}$ fresh weight), which equals 10- to 100-fold less than the amounts found in stressed naturally salt-tolerant plants. This suggests that if a method can be developed for accumulating high concentrations of glycinebetaine in a plant it could possibly lead to the development of a practical plant with high resistance to temperature or salt stress or, indeed, a wide range of other environmental stresses.

MEMBRANE LIPIDS

Lipids, which are among the basic constituents of biomembranes, have been a focus of attention since the 1960s as one of the factors affecting temperature sensitivity in plants (78). For example, the physiochemical characteristics displayed by lipid bilayers at different temperatures differ with the species of the lipid head group or their esterified fatty acids, and their lipid constituents and fatty acid constituents change depending on the environmental growth temperature (53, 94, 105). There has also been an interest in the relationship of low-temperature tolerance to the biosynthesis and rearrangement of biomembranes in response to temperature. Since the late 1980s, a series of mutant strains with altered fatty acid constituents in

their biomembranes have been isolated in *Arabidopsis*, providing a breakthrough in the clarification of the pathways by which the polyunsaturated fatty acids contained in biomembrane lipids are generated (95). In the 1990s, cloning the mutated genes, one after the other, using genetic approaches (7, 18, 23, 29, 77) became possible, and useful information such as the structure of the enzymes in the biosynthetic pathway for membrane lipids became available (90). The creation of transgenic plants by introducing these cloned genes also allowed the artificial modification of the fatty acid constituents of the biomembrane lipids and enabled the physiological significance of biomembrane lipids in temperature acclimatization to be clarified more directly (20, 38, 39, 60, 62, 64).

Phosphatidylglycerol

Artificially created lipid bilayers undergo a phase transition from a highly fluid liquid crystalline phase to a more solid gel phase following a temperature decrease. At low temperatures, biomembranes also have reduced liquidity and phase transitions; ion leakage and deactivation of membrane proteins both take place. These physical characteristics enabled research into the relationship between the fatty acid composition of biomembrane lipids and low-temperature sensitivity in plants. Murata (63) and Murata et al. (65) found that the levels of 16:0 and t16:1 fatty acids in phosphatidylglycerol (PG) in leaf tissue were high in chilling-sensitive plants but low in chilling-resistant plants. PGs from the chilling-sensitive plants changed from the liquid crystalline state into the phase separation state at approximately 30°C, whereas PGs from the chilling-resistant plants went into the phase separation state at 15°C or lower, a significant difference (66). In general, PG makes up 8–10% of chloroplast lipids, and most of this lipid is found in thylakoid membranes. In chilling-resistant plants, the amount of PGs with saturated fatty acids such as 16:0, t16:1, and 18:0 (saturated PGs) is less than 20%. By contrast, most chilling-sensitive plants contain 40% or more saturated PGs (35, 74, 83). These observations suggest that the saturated PG content in the chloroplast membranes might be related to the phase transition temperature, which is related to the low-temperature adaptability of plants.

Chloroplast PG always contains 16:0 or t16:1 in the sn-2 position. In chilling-resistant plants, the unsaturated fatty acid oleic acid (18:1) tends to bind in the sn-1 position, whereas in chilling-sensitive plants, the saturated fatty acid palmitic acid (16:0) tends to bind there. These differences result from the substrate selectivity of glycerol-3-phosphate-acyltransferase. Consequently, it is currently hypothesized that the difference in their sensitivity to chilling is caused by the properties of single enzymes (64). Experiments have been conducted to overexpress the genes for glycerol-3-phosphate-acyltransferase in tobacco, in an attempt to clarify the relationship between chilling sensitivity in plants and the fatty acid molecular species in PG (64). In transgenic tobacco overexpressing the acyltransferase gene (pSQ) from squash, a plant with chilling sensitivity, the content of the saturated PGs rose significantly to 76%, compared to the 36% found in wild-type tobacco,

whereas in transgenic tobacco overexpressing the same enzyme gene (pARA) from *Arabidopsis*, a plant with chilling resistance, the saturated PG content dropped slightly to 28%. After the plants were grown for 10 days under strong illumination, equivalent to approximately 7% sunlight, at 1°C, then returned to 25°C, the extent of chlorosis in the leaf tissue was the greatest in pSQ, followed by the wild-type strain, and with the least in the pARA strain.

Conversely, Wolter et al. (106) introduced the *E. coli* gene for glycerol-3-phosphate-acyltransferase (*plsB*), which is selective for saturated acyl-ACP as compared to unsaturated acyl-ACP, into *Arabidopsis* and expressed it in the chloroplasts. The saturated PG content in the chloroplast membranes of the transgenic *Arabidopsis* rose to over 50%, compared with that of 5% in the wild-type strain. The chilling sensitivity of the transgenic tobacco also increased. These findings indicate that the level of chloroplast PG saturation is one of the determining factors of chilling sensitivity of plants.

Light is a prerequisite for photosynthesis, but it is also harmful to the photosynthetic apparatus. Photoinhibition of photosynthesis is a major stress, which is aggravated if strong light is combined with other stresses, such as low temperature. How the increase in the saturated fatty acid species in PG affects photoinhibition under low-temperature conditions has been studied using the tobacco pSQ line, which is susceptible to low temperatures (60). However, no discernible difference was found in the extent of light-induced inactivation of photoinhibition, even when chilling treatment was applied. This suggests that the level of saturated PG content has no effect on the sensitivity of the photosynthetic machinery under stress induced by low temperatures. The primary target for photoinhibition is thought to be the D₁ protein of the photosystem II (PSII) reaction center (5, 6). When this protein is damaged by the photochemical reaction, de novo synthesis of the protein occurs and the active PSII is recovered. Thus, unsaturation of PG may influence the efficiency with which the photoinhibition-damaged D₁ protein is removed from the PSII reaction center complex and replaced by a new D₁ protein (60).

Studies of the mutant strain *Arabidopsis fab1*, being defective in palmitoyl-ACP elongase and having, therefore, an elevated level of 16:0 in its cellular lipids, have provided an alternative view of the role of unsaturated PG. The saturated PG content of the wild-type *Arabidopsis* is approximately 9%, whereas this mutant contains approximately 43% saturated PG (108). This is a significant difference, even compared with the saturated PG content of plant strains with a chilling sensitivity. Even though the saturated PG content of this mutant strain is higher than that of chilling-sensitive plants such as cucumber, it still does not suffer marked chilling damage even when grown at a temperature (2°C) that would kill the cucumber. This suggests that the level of saturated PG is not the only determinant of the plant's capacity to tolerate low temperatures, but that there are also other factors involved. Incidentally, the *fab1* mutant strain undergoes chlorosis when it is grown for an extended period at 2°C and ultimately withers and dies. This suggests that a high content of saturated PG also has an effect on long-term growth at low temperatures.

Polyunsaturated Fatty Acids

Analyses of mutant strains of *Arabidopsis* have shown that polyunsaturated fatty acids in chloroplast membranes influence the size of the chloroplast and the formation of its membranes at low temperatures (28). The *fad5* mutant is deficient in the activity of a chloroplast ω -9 fatty acid desaturase and accumulates high levels of palmitic acid (16:0). Conversely, the *fad6* mutant is deficient in the activity of the chloroplast ω -6 fatty acid desaturase and accumulates high levels of 16:1 and 18:1 fatty acids. Both mutants show correspondingly reduced levels of polyunsaturated fatty acids in the chloroplast galactolipids. When young seedlings of these mutants and the wild type (grown for seven days at 22°C) were subjected to chilling treatment (5°C) for three weeks, the mutant strains showed noticeably more chlorosis than the wild type. The amount of chlorophyll also decreased to approximately half of that in the wild-type strain. The chloroplast membrane (thylakoid membrane) content in the mutant strains decreased, and the chloroplast was smaller. In contrast, when more mature plants were subjected to similar chilling treatment, no difference was observed between the wild type and the mutant strains in the amount of chlorophyll, chloroplast size, or membrane content. Conversely, in the *fad2* mutant, which is deficient in the activity of 18:1 fatty acid desaturase localized in the ER and thus has a marked decrease in the amount of polyunsaturated fatty acids in the extrachloroplast membrane lipids (47, 77), long-term culture (42 days) at 6°C inhibited growth, eventually causing plants to wither and die (58). These observations suggest that polyunsaturated fatty acids are required for low-temperature survival and that under chilling stress the polyunsaturated fatty acids in chloroplast membrane lipids contribute significantly more to the regular formation of chloroplast membranes than to maintaining their stability when mature.

The cyanobacterium gene with a broad specificity for the Δ 9 desaturases (*des9*) was introduced into tobacco (D9-1 line) (32). The 16:1c7 fatty acids found in higher plants are bound to the sn-2 position of the chloroplastic lipid monogalactosyldiacylglycerol (MGD), and they exist only in minute quantities as intermediates in the production of 16:3. The 16:1t3 fatty acid that is bound to the sn-2 position of PG has characteristics similar to those of the saturated 16:0 fatty acids. The 16:0 fatty acids bound to the sn-1 position of PG, and all other lipid species are not desaturated. The D9-1 line produced 16:1c9 fatty acids that are not found naturally in higher plants, and they accounted for over 10% of all fatty acids esterified to individual membrane lipids. When the wild-type plants grown at 25°C were chilled at 1°C for 11 days, they underwent chlorosis, but this did not occur in the D9-1 line subjected to the same treatment. When the seeds of both the wild-type and the D9-1 plants were germinated at 10°C and grown for 52 days, the wild-type seedlings turned pale green in color and showed damage from chilling, but the D9-1 seedlings maintained growth, similar to their response at 25°C. Notably, the increase in chilling resistance, obtained by the introduction of the glycerol-3-phosphate-acyltransferase gene, is limited only to the process of recovery from chilling damage (64), yet the D9-1 line seedlings also displayed resistance to stress from direct exposure to low temperatures and reduced the lower temperature limit for plant growth (32).

Trienoic Fatty Acids

In general, trienoic fatty acids (TAs) are present in the highest percentage among fatty acids in plant membrane lipids. TAs are polyunsaturated fatty acids that have three cis double bonds, and their content varies considerably according to the plant species and the environmental conditions. TAs are formed from dienoic fatty acids (DAs) (having two cis double bonds) through the activity of ω -3 fatty acid desaturase. This desaturase seems to be deeply embedded within biomembranes. Owing to the difficulty of characterizing it by conventional biochemical approaches, its gene has been cloned using genetic techniques, namely map-based cloning methods that used mutant strains of *Arabidopsis* (7, 29). The ω -3 fatty acid desaturase genes cloned thus far are divided into two types, one localized in the chloroplasts (*FAD7* and *FAD8*) (18, 29) and the other localized in the ER (*FAD3*) (7).

Kodama et al. (38, 39) reduced the DAs (16:2+18:2) and increased the TAs (16:3 + 18:3) in leaf tissue by overexpressing the *Arabidopsis FAD7* gene in tobacco. Evaluation of low-temperature tolerance of transgenic and wild-type plants indicated no discernible difference in the performance of mature plants, but very young seedlings revealed differences in low-temperature tolerance. Transfer of wild-type plants grown at 25°C to 1°C for seven days without going through an acclimation process and subsequent return to the original temperature environment resulted in growth inhibition and chlorosis in the young leaves. This kind of damage was not observed in the transgenic tobacco, in which the level of TAs in the leaf tissue had been increased. However, when the levels of TAs in phospholipids, which are the main constituents of extrachloroplastic membranes, were increased in tobacco by overexpressing the ER-localized ω -3 fatty acid desaturase gene (*NtFAD3*), no significant difference was observed between wild-type and transgenic tobacco plants in their resistance to chilling and freezing (20). Conversely, alleviation of chilling injury at the seedling stage (92) and improvement of the germination rate under a low-temperature environment (M. Komaki & T. Shimada, personal communication) were observed in a transgenic line of rice with increased levels of TAs in their extrachloroplastic membranes. Although these results, obtained from the analysis of transgenic plants into which *FAD7* and *FAD3* genes had been introduced, provide direct proof that TAs enhance tolerance to low temperatures, the effect of TAs on the enhancement of low temperature tolerance was relatively slight, and it might be limited to specific plant species, and their tissues or growth processes.

As indicated above, although the increase in TAs did not produce as great an improvement in low-temperature tolerance as was expected, the reverse concept, that a decrease in the level of TAs in the organism may increase the high-temperature tolerance of plants, was examined (62). In the study described (38, 39), the ω -3 fatty acid desaturase gene was linked to a potent expression promoter, such as the cauliflower mosaic virus 35S promoter, to increase the amount of enzymes produced within the plant. However, in parts of the transgenic tobacco plants that were created for this purpose, transgenic lines were found in which the expression of the intrinsic ω -3 fatty acid desaturase gene was cosuppressed by gene silencing (62).

The correlation between the TA content of the biomembranes and the ability of the plant to tolerate high temperatures was analyzed using the transgenic tobacco lines in which the activity of chloroplast-localized ω -3 fatty acid desaturase was suppressed by gene silencing. Although TAs in the chloroplast membrane lipids of these transgenic tobaccos were held at an extremely low level, the level of DAs increased in a manner corresponding to the decrease in the level of TAs. In addition, few changes were detected in the lipid molecular species of biomembranes, other than in the chloroplast membrane.

In plants grown at temperatures ranging between cool (15°C) and a more suitable growth temperature (25°C), there were no differences between the growth of two transgenic tobacco lines (T15 and T23) and that of the wild type. After germinating and cultivating these plants for 45 days at 25°C, the fresh weight of the aerial parts of the T15 and T23 plants was 489 ± 71 mg and 513 ± 88 mg, respectively, whereas the fresh weight of the aerial parts of the wild-type plants was 497 ± 43 mg. The fresh weight of the aerial parts of the T15, T23, and wild-type plants cultivated at 15°C for 45 days was 6.2 ± 1.4 mg, 6.9 ± 1.2 mg, and 6.6 ± 0.9 mg, respectively. These results in tobacco plants are consistent with the growth of the *Arabidopsis fad7fad8* mutant within the normal cultivation temperature range of 12°C to 28°C (53). Furthermore, at temperatures below 10°C, the growth of the two transgenic lines and the wild type were similarly suppressed.

Although there was no difference observed in growth between the transgenic tobacco lines and the wild type over the range of low temperatures up to the normal growth temperature, at high temperatures clear differences in growth were observed. For example, the fresh weight of the above-ground portion of plants, grown for 45 days following germination at 30°C, was 492 ± 81 mg, 445 ± 62 mg, and 399 ± 69 mg, respectively, for the T15 and T23 lines and the wild-type plants. At a higher temperature (36°C), marked differences in the growth of the transgenic tobacco lines and the wild type were observed. After cultivating plants at 36°C for 45 days, the fresh weight of the aerial parts of the T15 and T23 lines and the wild-type plants was 124 ± 49 mg, 123 ± 23 mg, and 13 ± 6 mg, respectively. Because growth of the transgenic lines continued to be uninhibited beyond 45 days at 36°C, the resistance to high temperature was not transient and thus unlike the protection conferred by induction of a heat shock protein (44). When the plants were exposed to a considerably higher temperature (47°C), the leaves of the wild type withered within two days, and after three days the plant bodies exhibited chlorosis that resulted in death. In contrast, although growth of the transgenic plants was inhibited, damage due to high temperature was avoided by the plant body. The plants then continued to grow when returned to a more suitable temperature (25°C).

Photosynthetic activity was measured to investigate the mechanism by which the improvement in high-temperature tolerance was obtained. Although photosynthetic activity in wild-type tobacco plants at high temperatures of 40°C and above decreased considerably, the decrease was mild in transgenic tobacco plants with decreased levels of TAs (62). When the chloroplast membrane was studied using

differential scanning calorimetry, an endothermic peak in the range of 40–45°C that accompanies the thermal denaturation of proteins was observed in wild type, but not in the transgenic lines with decreased levels of TAs (Y. Murakami & K. Iba, unpublished data). Transgenic lines may have improved thermal stability in those proteins or functions that are associated with chloroplast membrane lipids. Prime candidates on which such protection could work are the proteins constituting the photosynthetic machinery.

High-temperature tolerance might also be conferred on plants by increasing the level of molecular species of fatty acids with a higher degree of saturation than that of DAs. In fact, it has been reported that the saturation of thylakoid membrane lipids by mutations in fatty acid desaturation (1, 27, 41) or by catalytic hydrogenation (101) increases the thermal stability of the membranes. Such increased saturation, however, could raise the temperature at which lipids, such as MGD, phase separate into nonbilayer structures, which disrupt membrane organization. Therefore, the sensitivity of such plants to low temperatures might be increased (28).

Regulation of Polyunsaturated Fatty Acid Levels by Temperature and Other Stresses

The growth rates of transgenic tobacco in which the TA level was altered did not differ significantly from that of wild-type plants (62). This was true at normal cultivation temperatures and even at the lowest temperature (15°C). These observations suggest that the reduction of the TA level might be able to confer high-temperature tolerance to plants without sacrificing their tolerance to low temperatures. However, within lower temperature ranges, especially near freezing, other factors such as a decrease in photosynthetic activity (52), growth inhibition, and chlorosis (28) might affect the plant's ability to withstand stress. Thus, alterations in the composition of membrane lipids might be a double-edged sword: That is, tolerance to either low or high temperatures, but not both, can be enhanced. Thus, if this technique is to be used to allow basic research on plant temperature-stress tolerance, clarifying how the TA level is regulated in conjunction with environmental change will be essential.

The expression of the chloroplast FAD8 ω -3 desaturase gene changes in response to a change in ambient temperature, whereas the expression of FAD7, a second chloroplast ω -3 desaturase, is not affected by temperature. With the *Arabidopsis fad7* mutant, deficient in FAD7 desaturase activity, the only activity of chloroplast ω -3 fatty acid desaturase that can be measured is that of the FAD8 desaturase (13). This makes it possible to clearly monitor the temperature dependency of the enzyme expression. Surprisingly, the expression of the FAD8 desaturase is switched on and off by a difference of as little as a few degrees Celsius on either side of 25°C (18). This suggests that this temperature regulation operates via a mechanism that is quite different from that governing the expression of temperature-dependent genes, such as the HSP genes, found so far. Recently, Matsuda & Iba (50) constructed a series of chimeric genes, created from both the

FAD7 and *FAD8* genes that encode the isozymes of chloroplast ω -3 desaturase, and introduced them into the *Arabidopsis fad7fad8* double mutant. Analyses of these transgenic plants showed that the temperature-dependent expression of the *FAD8* gene was due, not to the 5' flanking region, including the promoter region and the untranslated region, but to the exon/intron structure that is inherent to the *FAD8* gene. It therefore seems unlikely that *FAD8* gene expression is simply regulated at the transcriptional levels, as in the bacterial desaturase genes (99).

In the root tissues of wheat, the levels of 18:3 increase markedly at low temperatures. Conversely, in accordance with the increased accumulation of the ER ω -3 fatty acid desaturase protein, the mRNA level of the desaturase gene *TaFAD3* demonstrate minor change (26). This suggests that the increased level of 18:3 at low temperatures is regulated at the translational or posttranslational level.

The expressions of each of the ω -3 fatty acid desaturase genes, the chloroplast-localized (*FAD7*) and the ER-localized (*FAD3*) type, appear to be regulated in a complex way in response to changes in the environment or other stress-inducing factors. For example, environmental stimuli, such as wounding, salt stress, and pathogen invasion, lead to a rapid increase in a defense-related signal molecule, jasmonate. TAs, especially α -linolenic fatty acids, might play an important role as a precursor to jasmonate (16, 72), and the stimulus leads to a rapid induction of the expression of the chloroplast ω -3 desaturase genes (*FAD7* and *FAD8*) (21, 71, 73). In contrast, the expression of the ER ω -3 desaturase gene (*FAD3*) is regulated through the synergistic and antagonistic interaction of plant hormones such as auxin, cytokinin, and abscisic acid, and the tissue specificity of the expression of this gene is further modified in accordance with the growth phase in plant development (51, 110, 113). In view of these facts, the regulation of fatty acid desaturation of membrane lipids appears to be intimately related to the wide range of mechanisms that allow plants to adapt to their environment throughout development.

FUTURE PROSPECTS

Most approaches to molecular breeding have attempted to improve host resistance to temperature stress by introducing a single gene, thereby altering only a single character. However, the general view is that multiple characters are involved in tolerance over a wide range of temperatures. Consequently, approaches that aim to simultaneously alter multiple related characters are also being attempted in order to engineer better acclimation to freezing and chilling temperatures. *Arabidopsis* and tobacco transformed to express the transcription factors (activators) of those genes that play a role in acclimation have been generated. In such transgenic plants, intrinsic low-temperature-inducible genes are expressed selectively, and an enhancement of cold or freezing tolerance has been observed (33, 34, 36, 100).

Plants receive various stresses from their surrounding environment, which affect them in a complex manner. For example, when a plant is subjected to abiotic environmental stresses, such as high or low temperature, intense light, or drought,

its sensitivity to biotic stresses such as viruses and bacteria could be also intensified (17, 42). Previous studies of plant resistance to temperature stress have regarded temperature extremes as the sole source of stress and have not paid attention to the multiple stresses that affect populations under natural conditions. Even if tolerance to drought and salt stresses or tolerance to freezing was conferred on plants (34), for example, this addition would still keep the tolerance within only a single stress category because these stresses are mainly caused by a single effect of dehydration on cells. As a result, the development of a more effective approach to creating plants that can resist a wide range of stresses remains a challenge for the future. To enable us to address this issue, it is necessary to identify the key strategies that plants use to deal with complex stresses of both biotic and abiotic origin. For instance, the degree of unsaturation of fatty acids in the membrane lipids of plant cells is closely related to the plant's temperature tolerance (1, 27, 28, 32, 38, 39, 60, 62, 64, 101), but at the same time, it also influences the replication of viruses infecting the plant (46). This implies that the degree of unsaturation of lipid fatty acids could possibly determine whether a viral infection occurs. In future research, emphasis should be placed on such cases where tolerance to seemingly unrelated physical stresses of this kind and viral propagation are regulated by a single factor.

The swift development in recent years of functional genomics, the comprehensive systematic analysis of transcriptomics and proteomics, will enable us to understand the route by which signals that activate resistance to various stresses are transmitted (12, 48). By inferring the origins or intersections of such signals, we might be able to discover intrinsic resistance factors that are vital in allowing plants to cope with a wide range of stresses, including biological as well as physical and chemical environmental stress.

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