The Use of Bacterial Choline Oxidase, a Glycinebetaine-Synthesizing Enzyme, to Create Stress-Resistant Transgenic Plants[1]

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GLYCINEBETAINE (GB) AS A DETERMINANT OF STRESS TOLERANCE

GB is a zwitterionic, fully *N*-methyl-substituted derivative of Gly that is found in a large variety of microorganisms, higher plants, and animals (Rhodes and Hanson, 1993). At high concentrations, GB does not interfere with cytoplasmic functions and it efficiently stabilizes the structure and function of many macromolecules. Thus, it belongs to a group of compounds that are known collectively as compatible solutes.

GB appears to be a critical determinant of stress tolerance in plants. It is an extremely efficient compatible solute (Le Rudulier et al., 1984) and its presence is strongly associated with the growth of plants in dry and/or saline environments (Rhodes and Hanson, 1993). The accumulation of GB is induced under stress conditions (Gorham, 1995), and the level of GB is correlated with the degree of enhanced tolerance to stress (Saneoka et al., 1995). Exogenous application of GB improves the growth and survival of a wide variety of plants under various stress conditions (Allard et al., 1998; Hayashi et al., 1998). Furthermore, GB is much more effective than other compatible solutes in the stabilization in vitro of the quaternary structure of enzymes and complex proteins, as well as the highly ordered state of membranes, at high concentrations of salts and extreme temperatures (Gorham, 1995; Papageorgiou and Murata, 1995). These properties of GB were deduced for the most part from studies based on comparative physiology and genetics, as well as from experiments in vitro. However, such studies have in fact provided only circumstantial evidence for the important role in vivo of GB in the stress tolerance of plants.

A full understanding of the role of GB requires more than circumstantial evidence, and genetic engineering of unicellular cyanobacteria has provided a way for us to examine the physiological significance and the modes of action in vivo of this compatible/protective solute in the stress tolerance of photosynthetic organisms (Deshnium et al., 1995; Nomura et al., 1995). Similar transgenic approaches have proved fruitful in higher plants such as Arabidopsis, rice (*Oryza sativa*), and tobacco (*Nicotiana tabacum*), none of which normally synthesizes GB (Hayashi and Murata, 1998; Sakamoto and Murata, 2000). The aim of this *Update* is to summarize recent progress in experiments with transgenic phototrophs that has advanced our understanding on the role in vivo of GB in stress tolerance. Aspects of the physiology, biochemistry and genetics of the synthesis, and properties of GB have been covered elsewhere (Rhodes and Hanson, 1993; Gorham, 1995; McNeil et al., 1999).

CHOLINE OXIDASE IS A CONVENIENT ENZYME FOR GENETIC ENGINEERING OF THE BIOSYNTHESIS OF GB

All known pathways for the synthesis of GB start with choline and proceed through reactions that involve one or two enzymes for the oxidation of choline to GB (Hayashi and Murata, 1998). The one-enzyme reaction is catalyzed by choline oxidase (COD) in soil bacteria *Arthrobacter globiformis* and *Arthrobacter pascens*. The two-enzyme reaction is by a ferredoxin-dependent choline monooxygenase (CMO) and an NAD^+-dependent betaine aldehyde dehydrogenase (BADH) in the chloroplasts of higher plants. In mammalian cells and in microorganisms such as *Escherichia coli*, another two-enzyme reaction is catalyzed by an NAD^+-dependent choline dehydrogenase (CDH) and BADH.

Researchers had to choose one of the three distinct pathways to GB as the most suitable target for the genetic manipulations that would introduce a GB-biosynthetic pathway into non-GB-accumulating organisms. The COD pathway clearly has an advantage over the CDH/BADH and CMO/BADH pathways because a single transformation with the relevant gene should introduce the pathway from choline to GB, and COD does not require any cofactors for the catalysis. In fact, in studies of cyanobacteria and various plants, genetic engineering with the *codA* and *cox* genes, which encode COD of *A. globiformis* and *A.*

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pascens, respectively, has proved most successful, as indicated in Table I. Table I also shows that all the enzymes mentioned above have been expressed transgenically and, for the most part, constitutively in photosynthetic organisms that do not normally accumulate GB.

A general consequence of such transformation, which was not examined in all cases, is that the transgenic production of GB enhances stress tolerance to a moderate to significant extent, even though the accumulation of GB is osmotically insignificant (Table II). Such transgenic systems provide valuable models for the functional dissection of the role of GB in stress tolerance. In the following sections, we shall summarize the emerging view of how GB contributes in vivo to acclimation to a stressful environment.

TRANSGENIC CYANOBACTERIA

Accumulation of GB enhances the tolerance to high concentrations of NaCl of certain bacteria, e.g. *E. coli* (Le Rudulier et al., 1984). Furthermore, unicellular phototrophs can also acquire salt tolerance if transgenically produced GB accumulates in such cells, which do not normally produce this compatible solute. For example, *Synechococcus* sp. PCC 7942 (hereafter, *Synechococcus*) is a salt-sensitive, freshwater cyanobacterium that does not normally produce GB. As an initial step toward elucidation of the physiological importance in vivo of GB in the stress tolerance of photosynthetic organisms, members of two research groups almost simultaneously transformed this cyanobacterium with genes that are involved in the biosynthesis of this compatible solute (Deshnium et al., 1995; Nomura et al., 1995).

Transformation with the *codA* Gene Enhances Tolerance to High-Salt and Low-Temperature Stress

Synechococcus actively and efficiently incorporates and integrates exogenous DNA into its genome by homologous recombination. We introduced the *codA* gene for COD from *A. globiformis*, under the control of a constitutively active promoter, into the genome of the above-mentioned cyanobacterium (Deshnium et al., 1995). The resultant cells accumulated GB at levels as high as 80 mM when choline was supplied exogenously and the cells exhibited enhanced tolerance to salt stress, as evaluated in terms of growth, the accumulation of chlorophyll, and photosynthetic activity (Table II). They also exhibited enhanced tolerance to low-temperature stress (Table II; Deshnium et al., 1997). The phase transition of plasma membranes from the liquid-crystalline state to the gel state simultaneously shifted toward lower temperatures in the transgenic strain. This phenomenon

Table I. *Summary of transgenic cyanobacteria and plants engineered to synthesize GB*

Species	Enzyme/Gene[a] (Source)	Promoter	Subcellular Targeting	Maximum [GB][b]	Reference
Synechococcus	COD/*codA* (*A. globiformis*)	conII[c]	Cytoplasm	80 mM	Deshnium et al. (1995)
	CDH, BADH/*betA*, *betB* (*E. coli*)	Native	Cytoplasm	50 mM	Nomura et al. (1995)
Arabidopsis	COD/*codA* (*A. globiformis*)	35S	Chloroplast	1.2 μmol g^{-1} fresh wt	Hayashi et al. (1997)
	COD/*cox* (*A. pascens*)	2 × 35S	Cytosol	19 μmol g^{-1} dry wt	Huang et al. (2000)
O. sativa	COD/*codA* (*A. globiformis*)	35S	Chloroplast	1.1 μmol g^{-1} fresh wt	Sakamoto et al. (1998)
	COD/*codA* (*A. globiformis*)	35S	Cytosol	5.3 μmol g^{-1} fresh wt	Sakamoto et al. (1998)
	CDH/modified *betA* (*E. coli*)	35S	Mitochondrion	5.0 μmol g^{-1} fresh wt	Takabe et al. (1998)
N. tabacum	COD/*cox* (*A. pascens*)	2 × 35S	Cytosol	13 μmol g^{-1} dry wt	Huang et al. (2000)
	CDH/*betA* (*E. coli*)	35S	Cytosol	ND[d]	Lilius et al. (1996)
	CDH/modified *betA* (*E. coli*)	RbcS[e]	Cytosol	0.035 μmol g^{-1} fresh wt[f]	Holmström et al. (2000)
	CMO/not assigned (*Spinacia oleracea*)	35S	Chloroplast	0.05 μmol g^{-1} fresh wt	Nuccio et al. (1998)
Brassica napus	COD/*cox* (*A. pascens*)	2 × 35S	Cytosol	13 μmol g^{-1} dry wt	Huang et al. (2000)
Diospyros kaki	COD/*codA* (*A. globiformis*)	35S	Cytosol	0.28 μmol g^{-1} fresh wt	Gao et al. (2000)

[a] Transgenic expression of BADH alone is not included. [b] Maximum [GB], Maximum concentration of glycinebetaine. [c] conII, Constitutive promoter of *E. coli*. [d] ND, Not determined. [e] RbcS, Promoter of a gene for the small subunit of Rubisco from Arabidopsis. [f] 0.066 μmol g^{-1} fresh wt in transgenic progeny with both CDH and BADH.

Table II. *Summary of evaluation of stress tolerance of transgenic cyanobacteria and plants that synthesize GB*

Species	Enzyme[a]/Gene	Stage of Life Cycle	Type of Stress	Light Intensity	Evaluation of Tolerance	Reference
				$\mu E\ m^{-2}\ s^{-1}$		
Synechococcus	COD/*codA*	—	Salt (0.4 M NaC1/8 d)	70	Growth/PS[b]/CC[c,d]	Deshnium et al. (1995)
		—	Cold (20° C/6 d)	70	Growth[d]	Deshnium et al. (1997)
		—	Cold (20°C/2 h)	500	PS[d,e]	Deshnium et al. (1997)
	CDH, BADH/	—	Salt (0.4 M NaC1/3 d)	NA[f]	Growth[d]	Nomura et al. (1995)
	betA, *betB*	—	Salt (0.2 M NaC1/4 d)	NA	PS[d]	Nomura et al. (1995)
		—	Salt (0.3 M NaCl/4 d)	NA	CC[d]	Nomura et al. (1995)
Arabidopsis	COD/*codA*	Imbibition	Cold (0°C/2 h)	70	Germination[e]	Alia et al. (1998a)
		Imbibition	Heat (55°C/2 h)	70	Germination[e]	Alia et al. (1998b)
		Germination	Salt (0.3 M NaCl/8 d)	70	Germination[d]	Hayashi et al. (1998)
		Germination	Cold (10°C)	70	Germination[d]	Alia et al. (1998a)
		Germination	Heat (32.5°C)	70	Germination[d]	Alia et al. (1998b)
		Young	Salt (0.2 M NaCl/5 d)	75	Growth[d]	Hayashi et al. (1997)
		Young	Salt (0.1 M NaCl/20 d)	75	Survival[d]	Hayashi et al. (1997)
		Young	Cold (10°C)	70	Growth[d]	Alia et al. (1998a)
		Young	Heat (35°C/5 d)	70	Growth[d]	Alia et al. (1998b)
		Young	Heatshock (40°C/5 h)	70	Growth[e]	Alia et al. (1998b)
		Mature	Salt (0.2 M NaCl/10 d)	75	Growth[d]	Hayashi et al. (1997)
		Mature	Salt (0.4 M NaCl/48 h)	75	PS[d]	Hayashi et al. (1997)
		Mature	Cold (5°C/7 d)	250	VI[e,g]	Hayashi et al. (1997)
		Mature	Cold (5°C/4 d)	250	PS[d]	Hayashi et al. (1997)
		Mature	Freezing (−5°C/2 h)	Dark	Survival[e]	Sakamoto et al. (2000)
		Mature	Freezing (−10°C)	70	PS[d]	Sakamoto et al. (2000)
		Mature	Freezing (−6.3°C)	Dark	LT50[d,h]	Sakamoto et al. (2000)
		Mature	Strong light (3 h)	1,200	PS[d,e]	Alia et al. (1999)
	COD/*cox*	Young	Salt (0.1 M NaC1/23 d)	50	Growth[d]	Huang et al. (2000)
		Young	Drought	50	Growth[d]	Huang et al. (2000)
		Mature	Freezing (−5.6°C)	50	LT50[d]	Huang et al. (2000)
O. sativa	COD/*codA*	Young	Salt (0.15 M NaC1/7 d)	800	Growth[e]	Sakamoto et al. (1998)
		Young	Salt (0.1 M NaC1/23 h)	800	PS[d]	Sakamoto et al. (1998)
		Young	Cold (5°C/23 h)	1,300	PS[d]	Sakamoto et al. (1998)
	CDH/	Young	Salt (0.15 M NaC1/7 d)	650	Growth[e]/PS[d]	Takabe et al. (1998)
	modified *betA*	Young	Drought (<40% relative humidity/3 d)	650	Growth[e]	Takabe et al. (1998)
N. tabacum	COD/*cox*	Mature	Salt (0.15 M NaCl)	50	Growth[d]	Huang et al. (2000)
	CDH/*betA*	Young	Salt (0.3 M NaCl)	NA	Growth/VI[d]	Lilius et al. (1996)
	CDH/	Young	Salt (0.15 M NaC1/14 d)	Greenhouse	Growth[d]	Holmström et al. (2000)
	modified *betA*	Young	Salt (0.2 M NaC1/7 d)	390	PS[e]	Holmström et al. (2000)
		Young	Cold (4°C/1 h)	150	PS[d]	Holmström et al. (2000)
B. napus	COD/*cox*	Young	Salt (0.3 M NaC1/10 d)	290	Growth[d]	Huang et al. (2000)
		Young	Salt (0.3 M NaC1/10 d)	290	PS[d]	Huang et al. (2000)
		Young	Drought	290	PS[d]	Huang et al. (2000)
D. kaki	COD/*codA*	Young	Salt (0.1 M NaC1/48 h)	700	PS[d]	Gao et al. (2000)

[a] Subcellular localization of enzymes is described in Table I. [b] PS, Photosynthesis. [c] CC, Chlorophyll content. [d] Tolerance was evaluated under stress. [e] Tolerance was evaluated after removal of stress. [f] NA, Data not available. [g] VI, Visual inspection of injury. [h] LT50, Temperature at which 50% of electrolytes are released from tissues.

might be expected to be important to the enhanced tolerance of the cyanobacterium to low temperatures because the phase transition is accompanied with the loss of numerous functions of the membranes (Nishida and Murata, 1996).

Transformation with the *bet* Operon of *E. coli* Enhances Tolerance to Salt Stress

Nomura et al. (1995) took advantage of a prokaryotic gene expression system and transformed *Synechococcus* with a shuttle plasmid that carried the *bet* operon from *E. coli*. The operon contains four genes: the *betA* and *betB* genes for CDH and BADH, respectively; and the *betI* and *betT* genes that encode a putative regulatory protein and a protein that is involved in the transport of choline, respectively. The transgenic cyanobacterial cells accumulated GB at about 50 mM and contained increased levels of C-phycocyanin in response to salt stress. The activities of photosystems I and II exhibited enhanced stability, and the cells grew better than control cells

under salt stress (Table II). Nomura et al. (1998) also suggested that Rubisco might be a major target of damage by salt stress and that GB might protect this enzyme from salt-induced inactivation in the transgenic cells.

TRANSGENIC ARABIDOPSIS

Our successful genetic engineering of *Synechococcus* inevitably led us to apply the same strategy to higher plants that do not produce GB. Transformation of Arabidopsis with the *codA* gene from *A. globiformis* resulted in the accumulation of GB in various organs of transformed plants (Hayashi et al., 1997), and we were eager to examine the impacts of the accumulation of GB on the stress physiology of the transgenic plants at the cellular and the whole-plant level.

Transformation with the *codA* Gene Enhances Tolerance to Various Kinds of Stress at Various Stages of Development

Hayashi et al. (1997) produced Arabidopsis (ecotype Wassilewskija) plants that overexpressed COD under the control of the constitutively active 35S promoter of cauliflower mosaic virus. The *codA* gene was preceded by a sequence that encoded the transit peptide of the small subunit of Rubisco of tobacco. The maximum levels of GB accumulated were 1.2 μmol g^{-1} fresh weight and 18 μmol g^{-1} dry weight in shoots and mature seeds, respectively. Recent analysis suggests that considerable amounts of the GB is localized exclusively in the chloroplasts in the leaves (Sakamoto et al., 2000).

Table II summarizes the physiological responses to various stresses of transgenic Arabidopsis at various stages of development. The most striking effects of transformation were changes in the tolerance of high-salt stress. Transgenic Arabidopsis was tolerant to high-salt conditions during the germination of seeds, and the growth and survival of seedlings and mature plants under such conditions were markedly enhanced (Fig. 1; Hayashi et al., 1997, 1998). The transgenic plants were also tolerant to temperature stress over a broad range of temperatures. The transformation significantly enhanced tolerance to non-freezing low temperatures during the imbibition and germination of seeds, as indicated by higher frequencies and rates of germination than those of wild-type seeds (Alia et al., 1998a). Seedlings of transgenic plants had an increased biomass under low-temperature stress and mature plants were less susceptible to chilling-induced chlorotic damage (Hayashi et al., 1997; Alia et al., 1998a). The accumulation of GB also rendered the transgenic plants more tolerant to high-temperature stress (Alia et al., 1998b). Seeds of transgenic plants exhibited considerable tolerance to high temperatures during both imbibition and germination. Moreover, enhanced tolerance to high temperatures was not confined to the initial stages of development, as indicated by the accelerated growth and enhanced survival after heat treat-

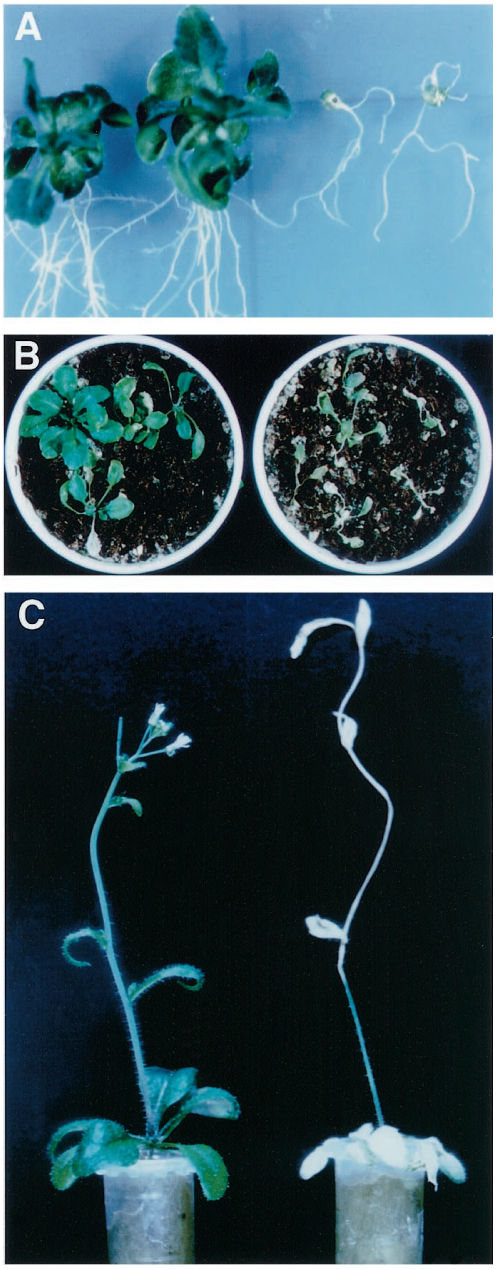

Figure 1. Enhanced tolerance to salt at various developmental stages of transgenic Arabidopsis that accumulates GB as a consequence of the introduction of the *codA* gene from *A. globiformis*. A, Seeds were germinated on gel-solidified medium supplemented with 100 mM NaCl and incubated for 20 d. The left two plants are transformed plants and the right two plants are wild type. Reproduced with permission from Hayashi et al. (1997). B, Two-week-old seedlings were exposed to 600 mM NaCl for 4 h, and after transferring to normal conditions they were allowed to grow for 3 weeks. Left, Transformed plants; right, wild-type plants. C, Thirty-day-old plants were watered with 200 mM NaCl for 10 d. Left, Transformed plant; right, wild-type plant. Reproduced with permission from Hayashi et al. (1997).

ment of seedlings of transgenic plants. One particularly interesting phenomenon, reported by Alia et al. (1998b), was that the extent of induction of a chloroplast-specific heat shock protein was lower at high temperatures in the transgenic plants than in the wild-type plants, indicating that GB might have reduced the effects of heat shock. At the other end of the temperature range, the accumulation of GB dramatically improved the survival of mature plants at freezing temperatures (Sakamoto et al., 2000). Several cold-regulated genes (*cor6.6*, *cor15a*, *cor47*, and *cor78*), which have been implicated in the development of freezing tolerance (Jaglo-Ottosen et al., 1998), seemed not to be responsible for the enhanced tolerance to freezing because there were no significant differences in the levels of their expression between wild-type and transgenic plants (Sakamoto et al., 2000).

We previously demonstrated that, in vitro, GB protects the photosynthetic machinery against salt and heat stress (Papageorgiou and Murata, 1995). The photochemical activity of photosystem II (PSII) in vivo, which we monitored in terms of changes in the fluorescence of chlorophyll *a* in intact leaves, was inhibited to a significantly lower extent in the transgenic plants than in wild-type plants under various types of stress, such as high concentrations of NaCl (Hayashi et al., 1997), low temperatures (Hayashi et al., 1997), and freezing temperatures (Sakamoto et al., 2000). The transformation also enhanced the tolerance to high-intensity light (Alia et al., 1999). It seems likely that this effect resulted from stimulation by GB, in chloroplasts, of the recovery of the damaged PSII complex, rather than from protection of the PSII complex against photo-induced damage (Alia et al., 1999).

Does Transformation with the *codA* Gene Have Harmful Effects?

Metabolic engineering of the synthesis of certain compatible solutes, such as sorbitol, has pleiotropic effects, for example necrosis and the development of growth defects, which are mostly due to disturbances in endogenous pathways of primary metabolism (Sheveleva et al., 1998). We were concerned that the overexpressed COD might compete for choline with the enzymes involved in the biosynthesis of phosphatidylcholine, a major component of membrane lipids. In fact, we found no significant differences in the respective levels of choline and phosphatidylcholine between transgenic and wild-type plants (Hayashi et al., 1997; Alia et al., 1999). Another concern was that COD might generate high levels of H_2O_2 as a by-product of the synthesis of GB. The level of H_2O_2 increased only modestly (1.5- to 2.0-fold) in transgenic plants under both stress and non-stress conditions, as compared with levels in non-transgenic plants (Alia et al., 1999). Simultaneously with the slight increase in the level of H_2O_2, we observed increased activation of ascorbate peroxidase and catalase, both of which detoxify H_2O_2 by converting it to water, in transformed plants as compared with wild-type plants (Alia et al., 1999). The H_2O_2 generated by choline oxidase seemed to induce the expression of these scavenging enzymes, with the resultant maintenance of intracellular H_2O_2 at a non-hazardous level.

Transformation with the *cox* Gene from *A. pascens*

The demonstration of the physiological impact of the overexpression of COD in chloroplasts was followed by an effort to determine the consequences of the overexpression of COD in a different subcellular compartment. Huang et al. (2000) transformed Arabidopsis (ecotype RLD) with the gene for COD, *cox*, from another soil bacterium, *A. pascens*. The accumulation of GB (maximum level, 18.6 μmol g^{-1} dry weight), which resulted from the expression of COD in the cytosol, was similar to that in the plants that overexpressed the enzyme in their chloroplasts (Table I). The level of GB increased still further when choline was supplied exogenously, indicating that the availability of substrate might limit the synthesis of GB when high-level accumulation of GB, similar to that in natural producers of GB, is the goal in non-accumulating species. In relative terms, the transgenic plants tolerated high-salt, drought, and freezing stress only moderately well (Table II). The marked differences between the results of transformation in this and the aforementioned studies indicate that differences in subcellular targeting of the biosynthetic enzyme might significantly affect the physiological impact of the accumulation of GB in intact plants.

TRANSGENIC RICE

Rice is not only a staple food worldwide but it is also an excellent model monocotyledonous plant. Rice plants are sensitive to various environmental stresses and they do not synthesize GB. The relative simplicity of systems for the transformation of rice and the refinement of systems for the expression of transgenes have enabled us to endow this important monocot with the ability to produce GB. Two research groups have succeeded in generating transformed rice that synthesizes GB, exploiting biosynthetic enzymes in different subcellular compartments (Sakamoto et al., 1998; Takabe et al., 1998), as summarized below.

Transformation with the *codA* Gene Enhances Salt and Cold Tolerance

We transformed the japonica variety of rice with two chimeric constructs in which the *codA* gene was

under control of the 35S promoter and which included an intron that originated in rice to enhance gene expression. One construct encoded COD with a signal for targeting to the chloroplasts, whereas the other lacked such a signal sequence, allowing COD to remain in the cytosol (Sakamoto et al., 1998). This study has been, to our knowledge, the only attempt to date to examine the effects of the subcellular compartmentalization of GB synthesis on stress tolerance of plants. The level of GB in leaves was 1 μmol g^{-1} fresh weight with the first construct and 3 to 5 μmol g^{-1} fresh weight with the second. The targeting of COD to the chloroplasts provided more effective protection of the PSII complex under high-salt and low-temperature conditions, suggesting that the subcellular localization of the synthesis of GB might be of crucial importance in the protection of the photosynthetic machinery against high-salt and cold stress. However, both types of transgenic rice grew better than wild-type plants during the recovery from salt stress (Table II).

Transformation with a Modified *betA* Gene Enhances Tolerance to High-Salt and Drought Stress

The CDH of *E. coli* catalyzes both the first and second reactions that convert choline to GB, although the latter reaction is less efficient than the former. Takabe et al. (1998) transformed japonica rice with the *betA* gene plus a signal for targeting to the mitochondria after modifications that eliminated possible polyadenylation signals, palindromic structures, and rarely used codons in rice, all of which might be expected to prevent the efficient expression of the sequence of interest. Transgenic plants that produced CDH accumulated betaine at levels as high as 5 and 1.2 μmol g^{-1} fresh weight in leaves and roots, respectively. By contrast, neither the native gene nor the modified gene without the targeting signal caused the accumulation of GB in transgenic plants, results that indicate that these genes were not efficiently expressed or, if they were expressed, that an appropriate cofactor (NAD^+) for the catalytic reaction was not available in the cytosol. Transgenic plants that accumulated GB survived better than control plants during the recovery from salt and drought stress (Table II). Furthermore, the photosynthetic machinery was more tolerant in transgenic plants than in wild-type plants to salt stress.

TRANSGENIC TOBACCO

Tobacco has been widely used as a model plant in efforts to identify genes responsible for stress tolerance and to improve the performance of plants under stress conditions. Genes for all the enzymes involved in the synthesis of GB from choline have been individually manipulated in tobacco (Lilius et al., 1996; Nuccio et al., 1998; Holmström et al., 2000; Huang et al., 2000). Thus, tobacco is the most intensively studied plant in terms of the possibility of engineering the synthesis of GB in a non-accumulator (see Table I). The various studies revealed the inefficient expression of transgenes and the remarkably low levels of accumulation of GB, as compared to those engineered in other species.

Transgenic tobacco expressing the *betA* gene from *E. coli* tolerated high-salt conditions, as determined by monitoring growth and biomass production (Table II; Lilius et al., 1996). However, the production of GB has not yet been confirmed in such transgenic tobacco. Therefore, the contribution of GB to the salt-tolerant phenotype of the transgenic plants remains to be clarified.

Another research group also introduced into tobacco the *betA* gene that had been modified so as to inactivate a possible poly(A) signal in the structural gene (Holmström et al., 2000). The resultant transgenic tobacco accumulated GB at an appreciable level, which was still considerably lower than that in transgenic Arabidopsis and rice (Table I). Transgenic tobacco that expressed the modified *betA* gene enhanced the biomass production under salt stress and the tolerance of the photosynthetic machinery to photoinhibition under salt and chilling stress (Table II). When this transgenic tobacco was crossed with transgenic tobacco that had been transformed to synthesize BADH, the resultant progeny produced both CDH and BADH, and accumulated nearly 2-fold higher levels of GB than transgenic plants with CDH alone (Holmström et al., 2000). However, it does not seem likely that the co-existence of CDH and BADH further improved the stress tolerance.

The potential for production of GB in tobacco by genetic engineering of the synthesis of CMO alone was examined by overexpressing the corresponding cDNA from spinach because tobacco has low BADH activity, although biochemical and molecular properties of such BADH have not been well characterized (Nuccio et al., 1998). Constitutive production and targeting to the chloroplasts of functional CMO resulted in very low levels of accumulation of GB in the transgenic plants (Table I) and, consequently, no change in phenotype was expected or recognized with respect to salt tolerance.

Transgenic tobacco expressing COD was generated by transformation with the same *cox* construct as had been used for transformation of Arabidopsis (Huang et al., 2000). While a few lines accumulated appreciable levels of GB (Table I), the vast majority of transgenic plants contained extremely low levels of COD and GB, if any. The phenotype of such transgenic lines strongly suggests the considerable limitations to the use of tobacco for genetic exploitation of the transgenic synthesis of GB.

MECHANISMS OF STRESS TOLERANCE: THE ROLE OF GB IN VIVO

Are the Protective Effects of GB Related to Osmotic Regulation?

Physiological studies of the functions of compatible solutes suggest that GB in the cell is most likely to act by reversing the osmotic imbalance between the intracellular and the extracellular environment that is caused by various types of stress. Such reversal can occur only when GB accumulates at high levels, such as the 400 μmol g^{-1} dry weight recorded in certain species under stress conditions (Rhodes and Hanson, 1993). By contrast, transgenic plants exhibit moderately or significantly enhanced stress tolerance even though levels of GB are low (up to 5 μmol g^{-1} fresh weight; Table I). Such low levels are insignificant in the context of osmoregulation. The concentrations of GB in transgenic *Synechococcus* (50–80 mM) were also insufficient to account osmotically for the acquired tolerance to as much as 400 mM NaCl. Moreover, this is not an isolated phenomenon because enhanced protection against stress has been observed in transgenic plants that produce low levels of other compatible solutes, such as mannitol (maximum level, 6 μmol g^{-1} fresh weight; Tarczynski et al., 1993) and trehalose (maximum level, 9 μmol g^{-1} dry weight; Holmström et al., 1996). Taken together, these studies suggest that GB has a protective rather than an osmotic effect in vivo, and they lead us to question the established view of GB as an osmoregulator exclusively.

Does GB Stabilize Complex Proteins and Membranes in Vivo?

The protective effects of GB on macromolecules, such as complex proteins and membranes, which have been well demonstrated in vitro, are clearly recognizable in vivo also. Under various stress conditions, GB protects the PSII complex from photoinduced inactivation in transgenic cells of *Synechococcus* and in plants (Table II). The protection by GB of the photosynthetic machinery against photo-induced damage can be attributed to acceleration of the recovery of the PSII complex from such damage (Deshnium et al., 1997; Alia et al., 1999; Holmström et al., 2000).

Evidence that membranes are targets of the action of GB in vivo has been obtained from *Synechococcus* cells that have been transformed with the *codA* gene (Deshnium et al., 1997). In the transgenic strain, there is a downward shift in the temperature at which the transition from the liquid-crystalline state to the phase-separated state occurs in the lipid phase of the plasma membrane. The lowering of the temperature of the phase transition is a critical mechanism in the enhancement of tolerance to low temperatures (Nishida and Murata, 1996). The unsaturation of fatty acids in membrane lipids has also been characterized well as a mechanism of acclimation that leads to low-temperature tolerance (Nishida and Murata, 1996). However, GB does not affect the unsaturation of fatty acids in *Synechococcus* (Deshnium et al., 1997) or in Arabidopsis (Alia et al., 1999), and this observation suggests that the enhancement of low-temperature tolerance by GB is due to the action in vivo of GB itself.

A role of GB in protecting membrane integrity was inferred from the enhanced tolerance to temperature stress that was observed during imbibition of seeds of transgenic Arabidopsis (Alia et al., 1998a, 1998b; Table II). Accumulated GB might prevent the destructive reorganization of membrane lipids when dry seeds are welted. Such reorganization often occurs when seeds are allowed to imbibe water at extreme temperatures. The results obtained from studies of transgenic plants and microorganisms reflect those obtained in a number of studies in vitro, with the exception that much higher concentrations of GB are required in vitro for reproduction of the protective effects of GB. This issue of concentrations remains to be resolved.

Does GB Protect the Transcriptional and Translational Machinery?

The fact that transgenic plants exhibited tolerance to various kinds of abiotic stress (notably Arabidopsis; Table II) suggests that GB might contribute to the maintenance of cellular functions of fundamental importance under stress conditions. There is some evidence for the involvement of GB in the protection of the transcriptional and translational machinery under stress conditions. Rajendrakumar et al. (1997) reported that GB decreases the melting temperature in vitro of double-stranded DNA. Such a destabilizing effect on DNA might facilitate replication and transcription in vivo in a high-salt environment. Eventually, Allard et al. (1998) observed that exogenous application of GB to wheat seedlings induced the expression of cold-inducible genes, suggesting that GB has an ability to enhance the transcription in vivo of genes that are involved in stress tolerance.

Processes involved in the gene expression are highly susceptible to various kinds of stress such as high concentrations of salt and extreme temperatures. GroEL, a chaperonin in *E. coli*, is effective in vivo in maintaining the function of the transcriptional and translational machinery by interacting with components of the machinery (Houry et al., 1999). Bourot et al. (2000) have recently demonstrated that GB behaves in vivo like the chaperonin. This seems to suggest that GB may stabilize the transcriptional and translational machinery for the efficient expression of genes under stress conditions. One mechanism that has been proposed as an explanation for the tolerance of transgenic Arabidopsis to high-intensity light is that GB might accelerate protein

synthesis de novo: the treatment with lincomycin, an inhibitor of protein synthesis in chloroplasts, diminishes the protective effect of GB (Alia et al., 1999).

How Important Is the Subcellular Localization of GB?

The subcellular localization of GB and the site of its biosynthesis seem to be crucial for efficient protection by GB from the effect of stress. The presence of enzymes that can catalyze the synthesis of GB in chloroplasts has been unequivocally demonstrated in Chenopodiaceae, but the occurrence of these enzymes in compartments other than chloroplasts cannot yet be ruled out in other plant families, such as Gramineae (McNeil et al., 1999). The accumulation of GB in chloroplasts has been demonstrated convincingly in natural accumulators of GB (McNeil et al., 1999), and such accumulation also seems to occur in transgenic plants when the appropriate enzyme is targeted to the chloroplasts (Sakamoto et al., 2000). Overexpression of COD in the chloroplasts had a more significant impact on the stress tolerance of transgenic plants than overproduction in the cytosol (Hayashi et al., 1997; Huang et al., 2000). For efficient protection of the photosynthetic machinery, it seems to be important to target the biosynthetic enzyme(s) to the chloroplasts (Sakamoto et al., 1998). From the available evidence, it seems reasonable to postulate that, in vivo, GB protects the physiological and metabolic activities of chloroplasts, in particular the photosynthetic machinery, by stabilizing the quaternary structures and functions of protein complexes such as the PSII complex. However, targeting of the enzyme for the biosynthesis of GB to mitochondria and the cytosol also enhanced the tolerance of the photosynthetic machinery to salt and chilling stress (Takabe et al., 1998; Holmström et al., 2000). The mechanism by which GB, synthesized in mitochondria or the cytosol, has a protective effect on the photosynthetic machinery remains to be elucidated.

PRACTICAL APPLICATIONS TO AGRICULTURE

Genetic engineering of the synthesis of GB and studies of the responses of transgenic plants to environmental stress have begun to provide some insight into the roles and functions in vivo of GB and the molecular mechanisms in stress tolerance. At the same time, the possibility has emerged that the same approaches might be used to improve the stress tolerance of agronomically important crops. From a biotechnological perspective, the initial focus has been on the further development of the capacity for synthesis of GB. With sophisticated control of the expression of transferred genes and enhancement of the availability of the substrate in a specific subcellular compartment, it may be possible to generate plants that produce elevated levels of GB and that are more tolerant than available cultivars to various stresses in the agricultural environment. However, such an approach might not be applicable to all crop species because the potential for the transgenic approach is limited in some species, e.g. tobacco (Huang et al., 2000). A second and extremely attractive approach is a combination of different strategies that are each, individually, effective in the enhancement of stress tolerance. Targets for this approach include genes involved in the biosynthesis of compatible solutes other than GB (Tarczynski et al., 1993; Holmström et al., 1996) and regulatory proteins, such as a stress-inducible transcription factor (Jaglo-Ottosen et al., 1998). After we have engineered the synthesis of GB, subsequent introduction of other genes that have been implicated in stress tolerance might have a significant impact on efforts aimed at increasing the stress tolerance of agriculturally important plants.

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