

Genetic engineering of glycine betaine biosynthesis to enhance abiotic stress tolerance in plants

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Abstract Glycine betaine (GB) is an important compatible solute that protects plants against the damaging effects of abiotic stresses. A number of plants have been engineered to contain genes of the GB biosynthetic pathway, which confers enhanced tolerance to a range of abiotic stresses during various plant developmental stages. Unlike natural accumulators, the transgenic plants accumulate very low GB concentrations, insignificant in terms of coping with osmotic stress. The GB accumulation in these transgenic plants varies depending upon their capacity for endogenous choline uptake, the type of gene that catalyzes the GB biosynthetic pathway, and the localization of the transgene product in a particular cellular compartment. This review focuses on recent progress in studies of abiotic stress tolerance conferred by GB in transgenic plants.

Key words: Abiotic stress, glycine betaine, transgenic plants, stress tolerance.

Abiotic stresses lead to a series of morphological, physiological, biochemical, and molecular changes that adversely affect plant growth and productivity (Wang et al. 2001). To ensure their integrity and survival, plants have evolved a number of defense strategies to cope with various abiotic stresses. One such approach adopted by plants to counteract osmotic imbalance is the production of osmoprotectants or compatible solutes (Yancey et al. 1982). Chemically, these are small, electrically neutral molecules and play important role in stabilizing proteins and membranes against the denaturing effects of abiotic stresses (Yancey 1994). Osmoprotectants or compatible solutes are of three types: (1) betaines (fully *N*-methylated amino acid derivatives), (2) proline and ectoine (amino acids), and (3) polyols and trehalose (non-reducing sugars).

Glycine betaine (GB), a quaternary ammonium compound, is one of the most efficient compatible solutes, and is found in a wide range of animals, bacteria, and some drought and salt-tolerant angiosperms (Rhodes and Hanson 1993; Chen and Murata 2002). Previously, it was proposed that the enhanced accumulation of betaine in plants plays an important physiological role of alleviating osmotic stress (Wyn Jones 1984). In natural accumulators such as spinach,

maize, sugar beet, and barley, rapid accumulation of GB occurs during exposure to salt, drought, and low-temperature stresses (Kishitani et al. 1994; Bohnert et al. 1995). GB protects plants by acting as an osmolyte, maintaining the water balance between the plant cells and the environment, and stabilizes macromolecules under cellular dehydration and high salt concentrations (Robinson and Jones 1986; Papageorgiou and Murata 1995).

Some economically important crops, including rice (*Oryza sativa*), potato (*Solanum tuberosum*), and tomato (*Solanum lycopersicum*), are unable to accumulate GB; therefore, these species are potential targets for engineering of betaine biosynthesis (McCue and Hanson 1990).

With increasing knowledge of genomics and proteomics coupled with gene engineering technologies, several plant species have been engineered with genes of the GB biosynthetic pathway that confer tolerance to several abiotic stresses (reviewed by Sakamoto and Murata 2002). In this review, we summarize recent progress in the genetic engineering of plants for GB biosynthesis, with particular emphasis on GB accumulation and abiotic stress tolerance.

Abbreviations: BADH, betaine aldehyde dehydrogenase; CDH, choline dehydrogenase; COD, choline oxidase; CMO, choline monooxygenase; dw, dry weight; fw, fresh weight; GB, glycine betaine; GSMT, glycine sarcosine methyltransferase; NADP, nicotinamide adenine dinucleotide phosphate; PSII, photosystem II; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; SDMT, sarcosine dimethylglycine methyltransferase; SIP, stress inducible promoter; UBI, ubiquitin promoter

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GB biosynthetic pathway

In general, in plants, animals, and microorganisms that are natural GB accumulators, the GB biosynthetic pathway starts with choline and progresses through a two-step dehydrogenation of choline and oxygenation of betaine aldehyde (an intermediate compound that is toxic to plants). In *Escherichia coli* and animals, the two-step pathway is catalyzed by choline dehydrogenase (CDH) and betaine aldehyde dehydrogenase (BADH), respectively (Takabe et al. 1998) (Figure 1A). In higher plants, choline is converted to GB through a two-step pathway catalyzed by choline monooxygenase (CMO) and BADH (Rathinasapathi et al. 1997) (Figure 1B). In contrast, GB biosynthesis in some microorganisms such as *Arthrobacter globiformis* and *Arthrobacter panescens* is accomplished by a single enzyme, choline oxidase (COD) (Ikuta et al. 1977) (Figure 1C). A new biosynthetic pathway of GB that begins with glycine as the precursor molecule has been discovered in two extremely halophytic microorganisms, *Actinopolyspora halophilia* and *Ectothiorhodospira halochloris* (Nyyssölä et al. 2000). In these

microorganisms, the GB biosynthetic pathway is accomplished by two enzymes: glycine sarcosine methyltransferase (ApGSMT) catalyzes the methylation steps from glycine to sarcosine (*N*-monomethylglycine) and sarcosine to dimethylglycine, and the other enzyme, sarcosine dimethylglycine methyltransferase (ApDMT), catalyzes the steps from sarcosine to dimethylglycine and dimethylglycine to betaine (Figure 1D).

Due to the immense importance of GB as an efficient compatible solute, research has mainly focused on the cloning and isolation of individual genes of the GB biosynthetic pathway in natural accumulators. Genes encoding CMO and BADH have been isolated from several higher plants (Rathinasapathi et al. 1997), CDH and BADH were cloned from *E. coli* (Landfald and Strom 1986), COX and COD were isolated from *Arthrobacter panescens* and *Arthrobacter globiformis* (Rozwadowski et al. 1991; Deshniem et al. 1995), and ApGSMT and ApDMT were isolated from *Actinopolyspora halophilia* and *Ectothiorhodospira halochloris*, respectively (Nyyssölä et al. 2000). Following their isolation from their natural accumulators,

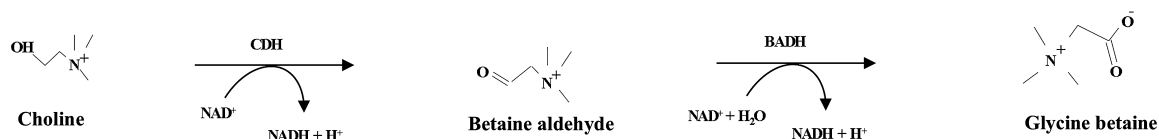
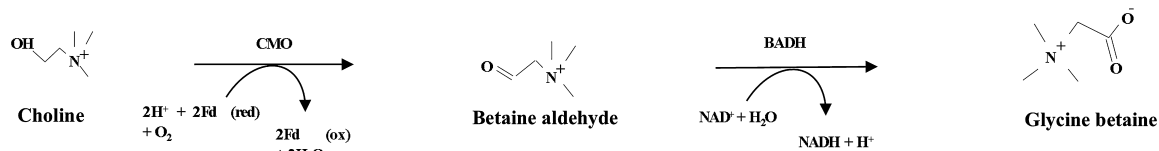
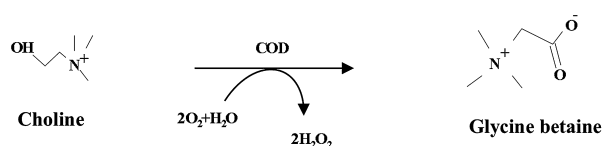
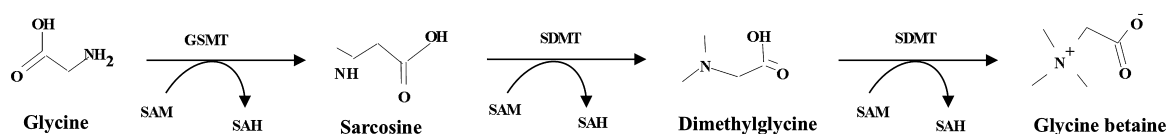
A *Escherichia coli***B** Plants**C** *Arthrobacter globiformis***D** *Actinopolyspora halophilia*

Figure 1. The GB biosynthetic pathways in different organisms. (A) The choline oxidation pathway in *Escherichia coli* (Takabe et al. 1998), (B) Plants (Rathinasapathi et al. 1997), (C) *Arthrobacter globiformis* (Ikuta et al. 1977), and (D) The Glycine methylation pathway in *Actinopolyspora halophilia* and *Ectothiorhodospira halochloris* (Nyyssölä et al. 2000).

these genes were engineered into various plants, resulting in increased tolerance to several abiotic stresses (Tables 1 and 2).

GB biosynthesis confers salt-stress tolerance

High salinity causes ion imbalance, toxic levels of cytoplasmic sodium, and osmotic stress (Ward *et al.* 2003). Plants have evolved two different types of

mechanisms to respond to salinity stress. Salt-tolerant plants accumulate excess salt in the vacuoles, controlling salt concentrations in the cytosol and maintaining a high cytosolic K^+/Na^+ ratio in their cells (Glenn *et al.* 1999), whereas salt-sensitive plants accumulate compatible solutes, which help to restrict the uptake of salt and adjust their osmotic pressure (Tal and Shannon 1983). In many plant species, GB accumulates in response to salt

Table 1. The accumulation of GB confers various stress tolerance.

Species	Salt	Low temperature	Drought	Heat	Strong light
<i>Arabidopsis thaliana</i>	+++ (1)	++ (2)	–	++ (2)	++ (3)
<i>Brassica napus</i>	+(4)	–	+(4)	–	–
<i>Daucus carota</i>	+++ (5)	–	–	–	–
<i>Diospyros kaki</i>	+(6)	–	–	–	–
<i>Solanum lycopersicum</i>	++ (7)	+++ (8)	–	–	–
<i>Nicotiana tabaccum</i>	++ (9)	–	–	++ (10)	–
<i>Oriza sativa</i>	+++ (11)	++ (12)	+(13)	–	–
<i>Solanum tuberosum</i>	++ (14)	–	+(14)	–	–
<i>Zea mays</i>	–	+(15)	++ (15)	–	–

‘+’ means low, ‘++’ means moderate and ‘+++’ means high level tolerance reported for that stress, while ‘–’ means no study is available so far. (1) Waditee *et al.* 2005; (2) Alia *et al.* 1998a, 1998b; (3) Alia *et al.* 1999; (4) Huang *et al.* 2000; (5) Kumar *et al.* 2004; (6) Gao *et al.* 2000; (7) Park *et al.* 2007a; (8) Park *et al.* 2004 (9) Yang *et al.* (10) Yang *et al.* 2005, 2007; (11) Mohanty *et al.* 2002; (12) Sakamoto *et al.* 1998; (13) Takabe *et al.* 1998; (14) Ahmad *et al.* 2008; (15) Quan *et al.* 2004a, 2004b.

Table 2. Metabolic engineering of plants for GB biosynthesis confers tolerance to various abiotic stresses.

Species	Gene	GB accumulation (organ)	Targeted organelle	Tolerance	References
<i>Arabidopsis thaliana</i>	<i>codA</i> (COD)	12.2–18.0 $\mu\text{mol g}^{-1}$ dw ^b (seeds)	chloroplast	cold	Alia <i>et al.</i> 1991a
<i>Arabidopsis thaliana</i>	<i>codA</i>	1.0 $\mu\text{mol g}^{-1}$ fw ^c (leaves/seeds)	chloroplast	chilling, salt	Hayashi <i>et al.</i> 1997, 1998
<i>Arabidopsis thaliana</i>	<i>codA</i>	12–18 $\mu\text{mol g}^{-1}$ dw (seeds)	chloroplast	heat	Alia <i>et al.</i> 1998b
<i>Arabidopsis thaliana</i>	<i>codA</i>	N.A. (shoots)	chloroplast	strong light	Alia <i>et al.</i> 1999
<i>Arabidopsis thaliana</i>	<i>codA</i>	0.70–0.90 $\mu\text{mol g}^{-1}$ fw (shoots)	chloroplast	freezing	Sakamoto <i>et al.</i> 2000
<i>Arabidopsis thaliana</i>	COX	19 $\mu\text{mol g}^{-1}$ dw (leaves)	cytosol	freezing, salt	Huanf <i>et al.</i> 2000
<i>Arabidopsis thaliana</i>	<i>GSMT/SDMT</i>	0.8–1.7 $\mu\text{mol g}^{-1}$ fw (seeds)	N.A. ^a	salt	Waditee <i>et al.</i> 2005
<i>Brassica juncea</i>	<i>codA</i>	0.82 $\mu\text{mol g}^{-1}$ fw (leaves)	chloroplast	salt	Prasad <i>et al.</i> 2000
<i>Brassica napus</i>	COX	13 $\mu\text{mol g}^{-1}$ dw (leaves)	cytosol	drought, salt	Huang <i>et al.</i> 2000
<i>Diospyros kaki</i>	<i>codA</i>	0.1–0.3 $\mu\text{mol g}^{-1}$ fw (leaves)	cytosol	salt	Gao <i>et al.</i> 2000
<i>Daucus carota</i>	BADH	93–101 $\mu\text{mol g}^{-1}$ dw (roots/leaves)	chloroplast	salt	Kumar <i>et al.</i> 2004
<i>Euchalyptus globulus</i>	<i>codA</i>	0.17–0.29 $\mu\text{mol g}^{-1}$ fw (leaves)	N.A.	salt	Yu <i>et al.</i> 2009
<i>Gossypium hirsutum</i>	<i>betA</i> (CDH)	130.2–142.0 $\mu\text{mol g}^{-1}$ dw (leaves)	N.A.	drought	Lv <i>et al.</i> 2007
<i>Nicotiana tabaccum</i>	COX	13 $\mu\text{mol g}^{-1}$ dw (leaves)	cytosol	salt	Huang <i>et al.</i> 2000
<i>Nicotiana tabaccum</i>	<i>betA</i>	≤66 nmol g ⁻¹ fw (N.A.)	cytosol	salt	Lilius <i>et al.</i> 1996
<i>Nicotiana tabaccum</i>	CMO (spinach)	0.02–0.05 $\mu\text{mol g}^{-1}$ fw (leaves)	chloroplast	salt	Nuccio <i>et al.</i> 1998
<i>Nicotiana tabaccum</i>	BADH (spinach)	0.44–4.92 $\mu\text{mol g}^{-1}$ fw (leaves)	chloroplast	salt	Yang <i>et al.</i> 2008
<i>Nicotiana tabaccum</i>	BADH (spinach)	0.46–4.6 $\mu\text{mol g}^{-1}$ fw (leaves)	chloroplast	high temperature	Yang <i>et al.</i> 2005
<i>Nicotiana tabaccum</i>	<i>BADH/SeNHXI</i>	4.7–7.0 $\mu\text{mol g}^{-1}$ dw (leaves)	N.A.	salt	Zhou <i>et al.</i> 2008
<i>Oriza sativa</i>	<i>betA</i>	5.0 $\mu\text{mol g}^{-1}$ fw (N.A.)	mitochondria	drought, salt	Takabe <i>et al.</i> 1998
<i>Oriza sativa</i>	<i>codA</i>	5.3 $\mu\text{mol g}^{-1}$ fw (leaves)	chloroplast/cytosol	chilling, salt	Sakamoto <i>et al.</i> 1998
<i>Oriza sativa</i>	<i>codA</i>	1.0–2.12 $\mu\text{mol g}^{-1}$ dw (leaves)	chloroplast	salt	Mohanty <i>et al.</i> 2002
<i>Oriza sativa</i>	COX	2.6–3.12 $\mu\text{mol g}^{-1}$ fw (leaves)	chloroplast	salt	Su <i>et al.</i> 2006
<i>Oriza sativa</i>	CMO (spinach)	0.29–0.43 $\mu\text{mol g}^{-1}$ dw (leaves)	chloroplast	salt	Shirasawa <i>et al.</i> 2006
<i>Solanum lycopersicum</i>	<i>codA</i>	0.1–0.3 $\mu\text{mol g}^{-1}$ fw (leaves)	chloroplast	chilling	Park <i>et al.</i> 2004
<i>Solanum lycopersicum</i>	<i>codA</i>	2.0 $\mu\text{mol g}^{-1}$ fw (reproductive organs)	chloroplast/cytosol	chilling, salt, oxidative	Park <i>et al.</i> 2007a
<i>Solanum tuberosum</i>	<i>codA</i>	0.9–1.43 $\mu\text{mol g}^{-1}$ fw (leaves)	chloroplast	oxidative, salt	Ahmad <i>et al.</i> 2008
<i>Zea mays</i>	<i>betA</i>	1.7–4.2 $\mu\text{mol g}^{-1}$ fw (leaves/seeds)	chloroplast	drought, chilling	Quan <i>et al.</i> 2004a
<i>Zea mays</i>	<i>betA</i>	2.6–4.0 $\mu\text{mol g}^{-1}$ fw (leaves)	chloroplast	drought	Quan <i>et al.</i> 2004b

^a ‘N.A.’ means not available. ^b dw, dry weight. ^c fw, fresh weight.

stress (Rathinasapathi et al. 2001). Physiological studies have demonstrated that the level of GB that accumulates is correlated with the degree of salt tolerance (Saneoka et al. 1995). Further studies showed that exogenously supplied GB increases salt tolerance in some plants that are non-accumulators of GB (Hayashi et al. 1998). In the last few years, several plant species have been engineered to contain genes of the GB biosynthetic pathway (Table 2). In spite of their low GB accumulation, the transgenic plants showed improved salt-stress tolerance (for a review, see Sakamoto and Murata 2002). For example, COD-transgenic *Arabidopsis* acquired salt-stress tolerance during seed germination and survived the damaging effects of elevated salt stress during the subsequent growth of seedlings and maturation of plants (Hayashi et al. 1997). Similar effects were observed in CDH-transgenic tobacco at comparatively low GB levels (Holmström et al. 2000). In addition, COD-transgenic plants, including rice, Japanese persimmon (*Diospyros kaki*), and *Brassica juncea*, showed high tolerance to salt stress (Sakamoto et al. 1998; Gao et al. 2000; Prasad et al. 2000a).

Rice plants transgenic for the bacterial CDH and COD accumulated the highest amount of GB reported from any plant species (Takabe et al. 1998; Sakamoto et al. 1998). Although the accumulation of GB was lower than in natural accumulators of GB, the transgenic rice plants showed enhanced tolerance to salt and drought stress. Improved salt tolerance was observed in COX-transgenic rice plants, which accumulated even lower amounts of GB than the CDH and COD-transgenic rice plants (Su et al. 2006). Similar results were reported in chloroplast-targeted CMO-transgenic rice plants (Shirasawa et al. 2006). These results indicate that although their GB contents are quite low, transgenic rice plants showed enhanced salt tolerance. It is possible that rice is more efficient in transporting choline from the cytosol to chloroplast, where the accumulated GB is more efficiently utilized to mitigate the damaging effects of salt stress on the photosynthetic machinery.

Salt-stress tolerance was achieved in chloroplast-targeted BADH-transgenic carrot (*Daucus carota*), which grew in the presence of up to 400 mM NaCl, a concentration at which only halophytes can survive, whereas the control plants showed severe growth retardation even at 200 mM NaCl (Kumar et al. 2004).

Recently, Waditee et al. (2005) demonstrated the co-expression of ApGSMT and ApDMT in *Synechococcus* and *Arabidopsis*. The transgenic *Synechococcus* and *Arabidopsis* accumulated higher levels of betaine than previously reported for choline-oxidizing enzymes. The transgenic *Synechococcus* thrived under high salinity, up to 0.6 M NaCl. As compared to choline oxidizing enzymes, GSMT and SDMT conferred greater salt tolerance on *Synechococcus*. The expression of choline-

oxidizing enzymes improved the salt tolerance of *Synechococcus* by approximately 0.03 M NaCl, whereas the co-expression of GSMT and SDMT improved it by about 0.2 M NaCl, from 0.35 up to 0.5–0.6 M NaCl. Transgenic *Arabidopsis* accumulated betaine in roots, stems, leaves, and flowers and showed improved seed yield under salt, drought, and low-temperature stresses. These results demonstrated the usefulness of the glycine *N*-methyltransferase genes for engineering the GB biosynthetic pathway into other abiotically stressed plants.

GB confers tolerance to drought stress

Water deficit or drought stress is one of the most important environmental constraints that limit agricultural production (Boyer 1982). Recent studies have revealed that both the exogenous application of GB and the genetic engineering of GB biosynthetic genes confer drought tolerance on plants. Quan et al. (2004b) reported drought tolerance in chloroplast-targeted CDH-transgenic maize plants. The transgenic maize showed improved drought tolerance at various developmental stages; most importantly, the grain yield of the transgenic plants was significantly higher than that of non-transformed control plants. The CDH-transgenic cotton plants showed improved drought-stress tolerance (Lv et al. 2007). The transgenic cotton plants accumulated up to 2.3–2.9 fold higher levels of GB than their non-transformed counterparts and showed significantly higher drought tolerance from the seedling to the flowering stage. Similarly, the potential usefulness of GB was observed in COD-transgenic rice plants (Sawahel 2003), which exhibited improved drought-stress tolerance. Moreover, several experiments revealed the positive effects of the exogenous application of GB on water-stressed plants. Similar effects were also observed in sunflower and tobacco plants under drought stress (Iqbal et al. 2005; Ma et al. 2007). GB application improved the water status and photosystem II (PSII) activity, resulting in increased biomass production in these plants.

Effect of GB on low temperatures

Low temperature is an environmental factor that limits the geographical distribution and growing season of many plant species, and it often adversely affects crop quality and productivity (Thomashow 1999). The damaging effects of low-temperature stresses include slow growth, low productivity, and even mortality of the plant (McKersie and Leshem 1994).

It has been proven that exogenous application of GB protects higher plants against low-temperature stress (Chen et al. 2000). The metabolic engineering of GB biosynthesis in a number of crop plants has resulted in enhanced tolerance to low temperatures at various stages

of plant development. In COD-transgenic *Arabidopsis*, cold-stress tolerance was achieved during imbibition and germination (Alia *et al.* 1998a). The transgenic plants showed higher frequencies of imbibition and accelerated rates of germination as compared to controls. Similar results were reported for COD-transgenic rice and BADH-transgenic tobacco plants (Sakamoto *et al.* 1998; Holmström *et al.* 2000).

In COD-transgenic tomato plants, GB showed an effect on chilling stress tolerance (Park *et al.* 2004). Despite very low GB accumulation, the transgenic tomato plants exhibited enhanced chilling tolerance at all developmental stages, from seed germination to the reproductive stage. The enhanced chilling-stress tolerance in the presence of very low GB levels may be due to the very low threshold level of endogenous GB ($>0.1 \mu\text{mol g}^{-1} \text{fw}$) that is sufficient to provide full protection against chilling stress in transgenic tomato (Park *et al.* 2004). In addition, both cytosolic and chloroplast-targeted COD-transgenic tomato plants showed a correlation between the accumulated GB in these compartments and the degree of tolerance to low temperature, salt, and oxidative stresses (Park *et al.* 2007a). The cytosolic-targeted COD-transgenic tomato plants accumulated five- to six-fold more GB than chloroplast-targeted COD-tomato plants. Despite low GB accumulation, enhanced tolerance to chilling, salt, and oxidative stresses was observed in the chloroplast-targeted COD-transgenic tomato plants.

Low-temperature stress induces cellular membrane dysfunction or protein denaturation, causing a disturbance in the electron transport system embedded in mitochondrial or chloroplastic membranes, and leading to the production of reactive oxygen species (ROS) (Nishiyama *et al.* 2001). High concentration of intracellular ROS has damaging effects on cellular components and also hinders repair of the PSII complex by inhibition of *de novo* protein synthesis (Nishiyama *et al.* 2001). Park *et al.* (2007a) suggested that one of the possible reasons for the greater protection against low-temperature stress in chloroplast-targeted COD-transgenic tomato plants might be an improved oxygen-evolving ability of the PSII complex by GB-induced repair. Another reason might be that GB stabilizes membrane integrity against extreme temperatures, reduces membrane lipid peroxidation, and protects PSII electron transport (Chen *et al.* 2000; Hamilton and Heckathorn 2001). Similar protective effects of GB were observed in CDH-transgenic maize plants (Quan *et al.* 2004a).

Another damaging aspect of low-temperature stress is that it induces the production of hydrogen peroxide (H_2O_2), which in turn activates the synthesis of catalases (Prasad *et al.* 1994). Both exogenous and endogenous application of GB enhances catalase activity and thus

enhances tolerance to low-temperature stress. An increase in catalase activity was observed in the chloroplast-targeted COD-transgenic tomato plants that demonstrated enhanced tolerance to both chilling and oxidative stress (Park *et al.* 2007a).

The GB localization in the chloroplast and the resulting enhanced tolerance to low temperatures is an indication that increased GB levels in the chloroplast are strongly correlated with PSII activity during chilling stress (Park *et al.* 2004). Likewise, in the chloroplast-targeted COD-transgenic tomato leaves, most of the GB accumulated in the chloroplast. Similar results were found in COD-transgenic rice and *Arabidopsis* (Sakamoto *et al.* 1998; Sakamoto *et al.* 2000). These findings suggest that the localization of GB in the chloroplast efficiently protects the photosynthetic machinery from the damaging effects of low-temperature stress.

GB protects photosynthesis against heat stress

Previous studies suggest that photosynthesis is a major target of high-temperature stress and PSII is the most temperature-sensitive component of photosynthesis (Berry and Björkman 1980). It has been demonstrated that PSII is highly affected by severe heat stress when temperature is above 45°C (Havaux 1996). Moderately high temperatures that normally inhibit CO_2 fixation have no lethal effects on PSII (Haldimann and Feller 2004). Severe heat stress normally affects several photosynthetic processes, while exposure to moderately high temperature impairs the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which appears to be the primary limitation to photosynthetic activity (Haldimann and Feller 2005). Several mechanisms are involved in the deactivation of the active sites of Rubisco and the deactivation process that leads to decreased photosynthetic rates, increases with increased temperatures (Salvucci and Brandner 2004). This phenomenon has been demonstrated through studies on cotton, tobacco, *Arabidopsis*, and wheat (Haldimann and Feller 2005).

Several *in vitro* studies have indicated that GB protects enzymes and protein complexes against heat-induced inactivation (Gorham 1995). GB plays a dual function by repairing the PSII complex during photoinhibition and protecting the complex proteins against heat-induced inactivation (Allakhverdiev *et al.* 2007). GB stabilizes the PSII complex by stimulating its repair when plants are exposed to cold and salt stress (Park *et al.* 2004). A similar mechanism might be involved during heat stress. COD-transgenic *Arabidopsis* that accumulated GB exhibited enhanced tolerance to high-temperature stress during the growth of young seedlings (Alia *et al.* 1998b). It is possible that the extreme-temperature tolerance induced in transgenic *Arabidopsis* is due to the

protection of Rubisco activase by GB. This finding was further supported by the increased photosynthetic activity in BADH-transgenic tobacco (Yang et al. 2005). The physiological basis for the enhanced tolerance of growth to high-temperature stress (25–45°C) in transgenic tobacco might be associated with an increased tolerance of photosynthesis to high temperatures. Moderately high temperatures do not affect the activity or efficiency of PSII, and the enhanced CO₂ assimilation rate induced by GB is associated with the Rubisco activase-mediated activation of Rubisco (Yang et al. 2005). The accumulation of GB increases the tolerance of Rubisco activase to high temperatures that results in enhanced tolerance of CO₂ assimilation in transgenic plants as compared to the wild type controls. In a recent study, the accumulation of GB in BADH-transgenic tobacco increased the tolerance of PSII to very high temperatures (up to 50°C) and improved the thermostability of the oxygen-evolving complex and the reaction center of PSII (Yang et al. 2007). It is thought that the increased tolerance of PSII to heat stress is associated with the prevention of the heat-induced photoinhibition of PSII.

GB protects photosynthesis from salt stress

Salt stress interferes with the intermolecular association of protein subunits, particularly the D1 protein of the PSII complex, whereas GB prevents the selective dissociation of the extrinsic polypeptides from the PSII complex in the presence of high concentrations of salts (Murata et al. 1992). In chloroplast-targeted COD-transgenic *Arabidopsis*, the extent of damage to the PSII complex was much lower than that observed in control plants under elevated salt concentrations (Hayashi et al. 1997). A similar study in *Synechococcus* indicated that GB mitigates the inhibitory effects of salt stress on the degradation and synthesis of the D1 protein during photoinhibition (Ohnishi and Murata 2006). In certain other plants, the expression of the *COD* and *CDH* genes revealed the same type of protection of the photosynthetic machinery under various abiotic stresses (Sakamoto et al. 1998; Takabe et al. 1998; Gao et al. 2000; Holmström et al. 2000; Prasad et al. 2000b).

Several studies have demonstrated that the improvement in photosynthesis by GB in salt-stressed plants is strongly correlated with enhanced PSII photochemical performance (Hayashi et al. 1997; Sakamoto et al. 1998; Holmström et al. 2000). Recently, Yang et al. (2005) reported that exogenous application of GB improved growth and CO₂ assimilation in maize plants under salt stress. Salt stress induced a significant decrease in the actual efficiency of PSII, whereas GB application resulted in a smaller decrease in the efficiency of PSII in salt-stressed maize plants. These results suggest that the improved photosynthetic CO₂

assimilation in salt-stressed maize is associated with improved PSII efficiency. On the other hand, salt stress induced a significant decrease in stomatal conductance, and GB application resulted in a lesser decrease in stomatal conductance in salt-stressed maize plants (Yang et al. 2005). Similar results were reported for transgenic tobacco plants, in which the accumulated GB alleviated the negative effects of salt stress on the photosynthetic machinery and conferred enhanced salt-stress tolerance on the growth of young seedlings (Yang et al. 2008).

Factors affecting GB accumulation in transgenic plants

Choline availability: Choline availability is one of the main factors that limits GB accumulation in transgenic plants (Huang et al. 2000). Previous studies revealed that both the exogenous and endogenous availability of choline can enhance GB accumulation (Sakamoto and Murata 2002). In CMO-transgenic tobacco, the accumulated GB level was very low (0.02–0.05 μmol g⁻¹ fw) under both normal and salt-stress conditions (Nuccio et al. 1998). However, transformation of CMO-tobacco plants with phosphoethanolamine *N*-methyltransferase increased the GB level by 30-fold (McNeil et al. 2001). In higher plants, choline biosynthesis occurs in the cytosol, and is then transported to the chloroplast, where GB biosynthesis occurs. It is possible that different plant species have different capacities for cytosolic choline biosynthesis and transport to the chloroplast for GB biosynthesis. COD-transgenic tomato plants accumulated more GB in the cytosol than the chloroplast, similar to CMO-tobacco (Park et al. 2007a). This indicates that both tobacco and tomato plants are less efficient at either choline biosynthesis and/or its transport to the chloroplast. However, COD-transgenic rice and CDH-transgenic maize accumulated the same levels of GB in the cytosol and chloroplast. These results suggest that the availability of endogenous choline and its transport from the cytosol to the chloroplast is species-specific and that both of these factors are crucial for increased GB accumulation and subsequent stress tolerance in transgenic plants.

Type of transgene: GB accumulation in transgenic plants partly depends on the type of transgene that catalyzes the GB biosynthetic pathway. The bacterial COD gene has proven to be more effective in GB accumulation than the other individual genes of the choline oxidation pathway. COD-transgenic *Arabidopsis* accumulated more GB than CMO-transgenic *Arabidopsis* (Hibino et al. 2002). Similarly, COD-transgenic tobacco accumulated GB more efficiently than CMO-transgenic tobacco (Nuccio et al. 1998; Huang et al. 2000). The highest GB accumulation in COD-transgenic rice clearly demonstrated the usefulness of *COD* for future transgenic efforts to develop tolerance

to abiotic stresses. The different levels of GB that accumulated in COD-, CDH-, and CMO-transgenic plants reveals that COD does not need to interact with any other gene to catalyze the GB biosynthetic pathway, but both CDH and CMO need endogenous BADH to complete the GB biosynthetic pathway. It is possible that the localization of the spinach CMO in the transgenic plants differs from that of endogenous BADHs. Shirasawa *et al.* (2006) suggested a similar mechanism for CMO-transgenic rice plants. CMO was targeted to the chloroplast using a transit peptide, whereas the endogenous BADH proteins were found in the peroxisomes. As a result of the localization of CMO and endogenous BADH in different cellular compartments, the CMO-transgenic rice accumulated very low levels of GB (Shirasawa *et al.* 2006). These studies suggest that the COD and CDH proteins do not interact with BADH-like enzymes that are localized in specific cytoplasmic compartments. It is also possible that the low level of GB accumulation in CMO-transgenic rice is due to the lower or inefficient catalytic activity of CMO as compared to the activities of COD and CDH (Shirasawa *et al.* 2006).

Promoter type: The type of promoter is another important factor that affects GB accumulation and the resulting stress tolerance. In some cases, beneficial effects of the transgene are masked by pleiotropic effects derived from the use of strong promoters (Cuartero *et al.* 2006). Recently, *COX* expression under the stress-inducible promoter (SIP) and a constitutive ubiquitin promoter (UBI) was reported in rice (Su *et al.* 2006). *UBI::COX* plants produced more GB ($3.12 \mu\text{mol g}^{-1} \text{dw}$) than *SIP::COX* plants ($2.60 \mu\text{mol g}^{-1} \text{dw}$). Under salt stress, the GB accumulation was enhanced up to 89% in the *SIP::COX* plants and only 44% in the *UBI::COX* plants. The extent of biomass production was significantly higher in *SIP::COX* plants than in *UBI::COX* plants. The effect of the *COD* gene under a stress-inducible promoter, SWPA2, has recently been reported in potato, and the resulting transgenic plants exhibited improved tolerance to oxidative, salt, and drought stresses (Ahmad *et al.* 2008).

Concluding remarks

Apart from its primary role in protecting vital enzymes and membranes during stress conditions, possible interactions between GB and other stress-related genes cannot be overlooked, as they may contribute, at least in part, to stress tolerance. The first clue of any such interaction between GB and other stress related genes came from the work of Allard *et al.* (1998). Exogenous GB was applied to two wheat cultivars and the accumulated GB conferred on the plants freezing stress tolerance. More importantly, the GB application resulted in the induction of several low temperature responsive genes, such as the *wcor410*, and *wcor413*. These genes

were also shown to be induced by salinity or drought stresses. Recently, Einset *et al.* (2007) reported the up-regulation of several genes in roots of GB-treated *Arabidopsis* plants under cold stress. These genes included the membrane trafficking *RabA4c*, the root-specific NADPH-dependent ferric reductase (FRO2), which localizes to the plasma membrane, mitochondrial catalase 2, and the cell wall peroxidase ATP3a. One of these genes, the membrane trafficking *RabA4c*, showed a ROS scavenging role in relation to GB, suggesting a possible interaction between oxidative stress, gene expression, and the accumulation of GB during stress conditions. There is also a possibility that the improved tolerance conferred by GB accumulation may have some relation with the expression of genes involved in other developmental processes. In one study conducted on *codA*-transgenic tomato plants, an interaction was found between GB accumulation, increased size of flowers and fruits and changes in the expression of genes involved in cell division (Park *et al.* 2007b).

Further efforts are required to concentrate on the chloroplast and mitochondrial genomes for GB biosynthesis, as previously enhanced tolerance to abiotic stresses has been achieved using these compartments. Genetic manipulation of the chloroplast genome may have several advantages such as high-level transgene expression, multi-gene engineering in a single transformation event, transgene containment via maternal inheritance and lack of gene silencing, and pleiotropic effects (Kumar *et al.* 2004). The mitochondrion is the center of ROS production, which is thought to be the main source of oxidative stress in plants. Engineering of the mitochondrial genome with genes of the GB biosynthetic pathway appears to be a promising approach toward unleashing the protective functions of GB against oxidative stress.

As abiotic stress tolerance is the result of polygenic expression in nature, efforts are required to combine different strategies involving multiple genes. The co-expression of Na^+/H^+ antiporters with genes of the GB biosynthetic pathway has the potential to increase salt tolerance by many times. Recently, co-transformation of the Na^+/H^+ antiporter gene (*SeNHX1*) and *BADH* was reported in tobacco (Zhou *et al.* 2008). The resulting transgenic plants accumulated more GB and showed higher biomass than either the single-gene transgenic plants or untransformed controls. Considering all of the factors described above, future research should be focused on the genetic engineering of economically important crop plants for enhanced and durable tolerance to multiple abiotic stresses.

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