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Dissecting the Role of Glycine Betaine in Plants under Abiotic Stress

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ABSTRACT

Among the compatible solutes, glycine betaine (GB) is particularly an effective osmolyte against abiotic stress. Metabolic acclimation of GB is regarded as a basic strategy for the protection and survival of plants in harsh environmental conditions. Plant species vary in their capacity to synthesize GB. Some plants, such as spinach and barley, accumulate relatively higher levels of GB in their chloroplasts while others, like Arabidopsis and tobacco, lack this compound. The accumulation of GB is induced under stress conditions and the level of GB is correlated with the degree of tolerance to stress. Genetic engineering has allowed the introduction of genes of its biosynthetic pathway into GB-deficient species from both microorganisms and higher plants. This approach has facilitated investigation of the importance of GB in stress protection. However, the level of GB in transgenic plants is relatively low. An alternative approach of exogenous application of GB to plants under stress has gained some attention. In this review, the protective role of GB in conferring tolerance to plants against various abiotic stresses through both exogenous application and genetic engineering has been summarized.

Keywords: compatible solute, drought, photosynthesis, antioxidants, reactive oxygen species, genetic engineering, transgenic plants
Abbreviations: **A**, net photosynthetic rate; **ApDMT**, *Actinopolyspora halophila* dimethylglycine methyltransferase; **ApGSMT**, *Actinopolyspora halophila* glycinesarcosine methyltransferase; **BADH**, betaine aldehyde dehydrogenase; **CAT**, catalase; **CDH**, choline dehydrogenase; **Chl**, chlorophyll; **CMO**, choline monooxygenase; **COD**, choline oxidase; **DW**, dry weight; **FW**, fresh weight; **GB**, glycine betaine; **H_2O_2**, hydrogen peroxide; **POX**, peroxidase; **PSII**, photosystem II; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase

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INTRODUCTION

Plant growth and productivity are adversely affected by nature's wrath in the form of various abiotic stress factors. Plants are frequently exposed to a plethora of stress conditions such as drought, flooding, salt, low temperature, heat, oxidative stress and heavy metal toxicity. When plants are exposed to stress factors, their cells protect themselves from high concentrations of intracellular salts by accumulating a variety of small organic metabolites that are collectively referred to as compatible solutes (Ashraf and Foolad 2007). Compatible solutes are very soluble in water and non-toxic even at higher concentrations. These metabolites allow cells to retain water and help in avoiding disturbances in their normal functions when exposed to abiotic stresses (Yancey *et al*. 1982). Compatible solutes include sugars, polyols, amino acids, proline and related compounds (Rhodes and Hanson 1993). One of the most-studied compatible solute is glycine betaine (*N,N,N*-trimethyl glycine, abbreviated as GB). GB is a zwitterionic, fully *N*-methyl-substituted derivative of glycine. It is found in a large variety of microorganisms, animals and higher

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Review

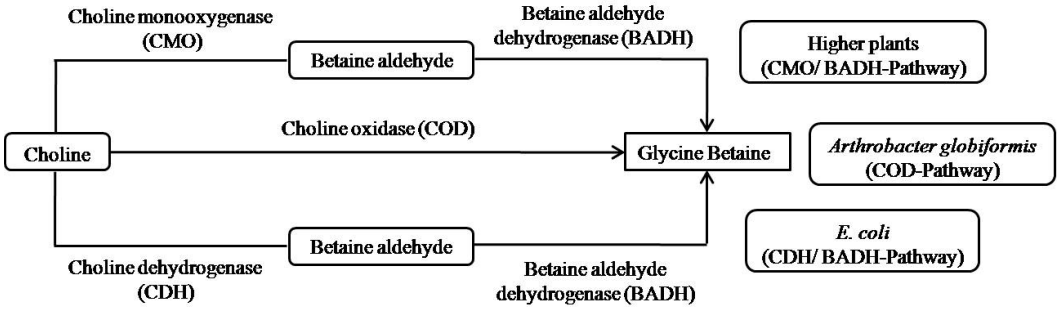

Fig. 1 Biosynthetic pathways of glycine betaine.

plants. It is dipolar but electrically neutral at physiological pH (Rhodes and Hanson 1993). GB is synthesized at elevated rates in response to abiotic stresses (Allard *et al*. 1998). Levels of accumulated GB are generally correlated with the extent of increased tolerance by plants (Rhodes and Hanson 1993). Many taxonomically distant plant species normally contain low levels of GB (these plants are known as natural accumulators of GB), but can also accumulate larger amounts of GB when subjected to abiotic stress. In many other species, GB has not been detected under normal or stressful conditions (natural non-accumulators) (Chen and Murata 2011).

There are many reports which suggest that the diverse roles played by GB in higher plants such as barley, maize, spinach and sugar beet (Chen and Murata 2008). GB effectively stabilizes the quaternary structures of enzymes and complex proteins and protects various components of the photosynthetic machinery, such as ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) and the oxygen evolving photosystem II (PSII) (Murata *et al*. 1992) and maintains highly ordered state of membranes at non-physiological temperatures and high salt concentrations (Papageorgiou and Murata 1995). Both the exogenous application of GB and also the introduction, via transgenes, of the GB-biosynthetic pathway into naturally non accumulators increase the tolerance of such plants to various types of abiotic stress. This increased tolerance to abiotic stress provides useful systems for investigating the mechanism by which GB protects plants against abiotic stress.

BIOSYNTHESIS OF GLYCINE BETAINE

GB is synthesized via two distinct pathways from two different substrates such as choline and glycine (**Fig. 1**). The biosynthesis of GB from choline has been studied in majority of biological systems, including animals, plants and microorganisms. This pathway involves one or two enzymes, depending on the mode of oxidation of choline. GB is synthesized as a result of the two-step oxidation of choline via betaine aldehyde, a toxic intermediate. The first step is catalyzed by choline monooxygenase (CMO) in higher plants, whereas the same step is catalyzed by choline dehydrogenase (CDH) in *Escherichia coli* and animals. The second oxidation step is catalyzed by NAD^+-dependent betaine aldehyde dehydrogenase (BADH) (Takabe *et al*. 2006). In contrast, GB is synthesized from choline by a single enzyme, choline oxidase (COD) in soil bacterium *Arthrobacter* sp. (Ikuta *et al*. 1977). The biosynthesis of GB is stress-inducible and the concentration of GB *in vivo* varies among plant species, ranging from 40 to 400 μmol g^{-1} (DW) in natural accumulators under stress conditions (Rhodes and Hanson 1993).

GENETIC ENGINEERING OF GLYCINE BETAINE BIOSYNTHESIS IN PLANTS

GB confers osmoprotection in bacteria, plants and animals, and protects cell components against harsh conditions, *in vitro*. Major cereals like wheat, maize and barley do not accumulate significant amount of GB naturally. This could be due to the production of truncated transcripts for GB biosynthesizing enzyme (BADH) (Giri 2011). Among these, rice is the only cereal that does not accumulate GB naturally as well as under stress conditions. Like rice, many crop plants such as potato, Arabidopsis, mustard, tobacco and tomato do not accumulate GB and are therefore potential targets for engineering the GB biosynthesis. Identification of genes of GB biosynthetic pathways has made it easy to engineer GB biosynthesis into non-accumulators by transgenic approach to enhance stress tolerance. This approach has been successfully used in diverse plant species, e.g., *Arabidopsis*, tobacco, *Brassica*, persimmon, tomato, maize, rice, and wheat to improve their abiotic stress tolerance (**Table 1**). GB accumulates at a high concentration (4-40 μmol g^{-1} FW) in naturally accumulating plants like spinach, sugar beet and act as osmoprotectant in abiotic stress conditions. However, transgenic plants carrying GB synthesizing genes produced much reduced amount of GB (0.05-5 μmol g^{-1} FW) (Giri 2011). It is more likely at these concentrations GB conferred protection of cellular macromolecules in transgenic plants.

Several reports have demonstrated that the metabolic engineering of plants leading to biosynthesis of GB have enhanced tolerance to drought (**Table 1**). Sawahel (2004) demonstrated the potential usefulness of GB in the improvement of drought tolerance of rice plants. GB content in leaves of transgenic rice plant under water stress was higher (4.1 ± 0.3 μmol g^{-1} FW) in comparison to non-stressed condition (1.5 ± 0.2 μmol g^{-1} FW). These results were comparable with the findings of Kishore *et al*. (1995) in transgenic proline-accumulating tobacco plants under water stress suggesting that GB accumulation in transgenic plants has a similar effect to the osmoprotectant proline in helping the cells to maintain osmotic potential and thus enhancing the ability of the plants to tolerate water stress. Moreover, under stress conditions, GB not only allows cells to adjust the osmotic potential in their cytoplasm to maintain appropriate water content, it also protects proteins from the stress-induced dissociation into their respective subunits.

Saneoka *et al*. (1995) demonstrated, through studies at the level of plant physiology and genetics, that the rate of accumulated GB is correlated with the degree of salt tolerance. To obtain direct proof that the accumulation of GB *in vivo* enhances the ability of plants to tolerate high concentrations levels of NaCl, studies were designed to assess the physiological consequences of the genetic engineering of GB synthesis via overexpression of two choline-oxidizing enzymes, namely CMO and CDH (Hayashi *et al*. 1998; Holmstrom *et al*. 2000). Transgenic *Arabidopsis* plants that produced CMO in their chloroplasts not only acquired resistance to high levels of salt during germination but were also able to tolerate high levels of salt during the subsequent growth of seedlings and mature plants (Hayashi *et al*. 1998). Genetic engineering of tobacco with a gene for CDH enhanced plant growth under salt stress, although the level of GB was much lower than that in CMO-engineered *Arabidopsis* (Holmstrom *et al*. 2000). In addition, *Brassica juncea* and Japanese persimmon (*Diospyros kaki*) have also been successfully transformed by introducing the gene for CMO to tolerate salt stress (Prasad *et al*. 2000).

Table 1 Transgenic cyanobacteria and plants engineered to synthesize GB and their enhanced tolerance to abiotic stress.

Species	Enzyme/gene	Subcellular localization	Maximum accumulation	Enhanced tolerance	Reference
Synechococcus	COD/*codA*	Cytoplasm	80 mM	Salt	Deshnium *et al*. 1995
	CDH, BADH/*betA*, *betB*				
	COD/*codA*	Chloroplast	50 mM	Chilling	Alia *et al*. 1998b
Arabidopsis thaliana	COD/*cox*	Cytosol	19 μmol g^{-1} DW	Salt+drought+freezing	Huang *et al*. 2000
	COD/*codA*	Chloroplast	0.82 μmol g^{-1} FW	Salt	Prasad *et al*. 2000
	COD/*codA*	Chloroplast	1.2 μmol g^{-1} FW	Salt	Sulpice *et al*. 2003
	ApGSMT+ApDMT [+5 mM glycine]	Cytosol	2 μmol g^{-1} FW	Salt+chilling+$CuSO_4$	Waditee *et al*. 2005
Brassica napus	COD/*cox*	Cytosol	13 μmol g^{-1} DM	Salt	Huang *et al*. 2000
Zea mays	BADH/*betA*	Cytosol	5.7 μmol g^{-1} FW	Chilling	Quan *et al*. 2004a
	BADH/*betA*	Cytosol	5.7 μmol g^{-1} FW	Drought	Quan *et al*. 2004b
Nicotiana tabacum	COD/*codA*	Chloroplast	2.12 μmol g^{-1} FW	Salt	Mohanty *et al*. 2002
	COD/*codA*	Chloroplast	0.43 μmol g^{-1} DW	Salt, day/night temp (28/13°C)	Shirasawa *et al*. 2006
Oryza sativa	COD/*cox*	Chloroplast	3.1 μmol g^{-1} DW	Salt	Su *et al*. 2006
Gossypium hirsutum	BADH/*bet A*	Cyotosol	354 μmol g^{-1} DW	Drought	Lv *et al*. 2007
Solanum tuberosum	COD/*codA*	Chloroplast	1.4 μmol g^{-1} FW	Osmotic+salt+drought	Ahmad *et al*. 2008
Lycopersicon esculentum	COD/*codA*	Chloroplast	95 nmol mg^{-1}chlorophyll	Oxidative	Park *et al*. 2007
	COD/*codA*	Chloroplast	23 nmol mg^{-1} chlorophyll	Chilling+salt+oxidative	Park *et al*. 2007
	COD/*codA*	Chloroplast and cytosol	1 μmol g^{-1} DM	High temperature	Li *et al*. 2011
Triticum aestivum	BADH/*betA*	Chloroplast	170.7 μmol g^{-1} DW	Drought	Wang *et al*. 2010
	BADH/*betA*	Chloroplast	140.2 μmol g^{-1} DW	Heat	Wang *et al*. 2010
Ipomoea batatas	BADH/*betA*	Chloroplast	-	Salt+low temperature+oxidative	Fan *et al.* 2012

High temperature stress is one of the main factors that limit the growth and productivity of plants (Frova 1997). Living organisms respond to high temperatures by metabolic changes that involve complex reprogramming of cellular activities and are evidently needed to protect the essential structures and functions of cells against the damage caused by the stress. Several *in vitro* studies have indicated that GB may enhance tolerance of plants to high temperature. GB is in particular effective in protecting some enzymes and protein complexes such as PSII complex against heat-induced inactivation (Allakhverdiev *et al*. 1996). An *in vivo* study has further shown that the transformed *Arabidopsis* with an accumulation of GB exhibits enhanced tolerance to high temperatures during the growth of young seedlings (Alia *et al*. 1998a). Inhibition of photosynthesis by high temperature stress is common for plants in tropical and subtropical regions and the temperate zones where plants are exposed periodically to high temperatures (Larcher 1995). PSII has long been considered the most heat-sensitive component of the photosynthetic apparatus for many years (Berry and Bjorkman 1980). However, it appears that PSII is unaffected at temperatures that inhibit CO_2 fixation (Weis 1981) and that PSII is only inhibited when the temperature is higher than 45°C (Havaux 1996). Recent studies seem to support that PSII activity is not limiting at temperatures that inhibit CO_2 fixation and that CO_2 fixation is most sensitive to heat stress due to the inhibition of activation of Rubisco via a direct effect on Rubisco activase (Haldimann and Feller 2004). Yang *et al*. (2005) have demonstrated that the physiological basis for enhanced tolerance to high temperatures may be associated with induced accumulation of GB, *in vivo*. They established that transgenic tobacco plants with the ability to synthesize GB by introduction and overexpression of a gene for BADH from spinach. This manipulation enabled the plants to accumulate GB mainly in chloroplasts and resulted in enhanced tolerance to high temperature stress during growth of young seedlings. Moreover, CO_2 assimilation of transgenic plants was significantly more tolerant to high temperatures than that of wild-type.

Yang *et al*. (2005) further analyzed the chlorophyll fluorescence and the activation of Rubisco which indicated that the enhancement of photosynthesis to high temperatures was not related to the function of PS II but to the Rubisco activase-mediated activation of Rubisco. Western-blotting analyses showed that high temperature stress led to the association of Rubisco activase with the thylakoid membranes from the stroma fractions. However, such an association was much more pronounced in wild-type plants than in transgenic plants. The results in this study suggest that under high temperature stress, GB maintains the activation of Rubisco by preventing the sequestration of Rubisco activase to the thylakoid membranes from the soluble stroma fractions and thus enhances the tolerance of CO_2 assimilation to high temperature stress.

Genetic engineering of *Arabidopsis* that resulted in the expression of COD significantly also increased the tolerance to low temperatures at various stages of development. The seeds of transgenic plants were more tolerant to low temperatures during imbibition and germination with higher frequencies and accelerated rates of germination respectively than controls (Alia *et al*. 1998b). The production of biomass by both young and mature transgenic *Arabidopsis* plants was also enhanced at low temperatures in comparison to controls (Hayashi *et al*. 1997).

It has been reported that GB has a cryoprotective effect *in vitro* on enzymes and membranes. Sakamoto *et al*. (2000) have shown that the transformation of *Arabidopsis* with the *codA* gene for COD enhanced freezing tolerance significantly when tolerance was evaluated in terms of viability and the retention of intracellular ions after freezing treatments. Cold-regulated proteins, which have been implicated in the development of freezing tolerance (Jaglo-Ottosen *et al*. 1998), did not seem to be responsible for the enhanced freezing tolerance of these transgenic plants, because the level of these proteins was unaffected by the transformation.

ACCUMULATION OF GLYCINE BETAINE

Site of glycine betaine accumulation

The tolerance of plants to abiotic stress is influenced by two main factors that are the concentration and the localization of GB in cells. There are many reports of the engineered accumulation of GB in plants in which GB-biosynthetic enzymes have been targeted to chloroplasts. However, there are few studies shows that the enzymes have been targeted to either cytosol or mitochondria, or to both cytosol and chloroplasts simultaneously (**Table 1**).

In *codA*-transgenic plants, the gene product has been targeted to chloroplasts (Chen and Murata 2002). The resultant transgenic plants accumulate GB primarily in their chloroplasts and they exhibit tolerance to various abiotic

stresses (Park *et al*. 2004). For instance, Sakamoto *et al*. (1998) generated transgenic rice plants using *codA* gene that was targeted to chloroplasts and found that photosynthetic machinery was protected against salt stress and cold stress more efficiently than it was in rice plants transformed with a cytosol-targeted *codA* gene, even though the transgenic plants of cytosol-targetd *codA* accumulated fivefold higher levels of GB in leaves. Three types of transgenic tomato plants were further produced by Park *et al*. (2007) using *codA* gene that was targeted to chloroplasts (Chl-*codA* plants), to the cytosol (Cyt-*codA* plants), or both chloroplasts and cytosol simultaneously (Chl Cyt-*codA* plants). Cyt-*codA* and Chl Cyt-*codA* plants accumulate up to 5.0- and 6.6-fold, respectively, higher levels of GB in their leaves than Chl-*codA* plants that accumulated 0.3 μmol g^{-1} FW. They found that all the three types of transgenic plants exhibited greater chilling tolerance than wild type plants. The stress tolerance during the growth of seedlings of Chl-*codA* plants was higher, even though the level GB was the lowest than those of others. Moreover, the stress tolerance of PSII and the frequency of seed germination were similar to that of other two types of transgenic plant. These observations lead to conclusion that the accumulation of GB in chloroplasts is more effective than the accumulation of GB in the cytosol for the protection of plants against abiotic stress.

Choline supplementation enhances glycine betaine accumulation

Rice plants do not accumulate GB naturally, but the highest levels of accumulated GB have been found in leaves of *codA* transgenic rice plants (53 μmol g^{-1} FW; Sakamoto *et al*. 1998). While in a natural GB-accumulator maize (*Zea mays*), the highest reported level of GB in leaves of *bet-A* transgenic maize plants is 5.7 μmol g^{-1} FW (Quan *et al*. 2004b). Thus, in terms of maximum levels of GB in transgenic plants, there appears to be no significant difference between plants that accumulate GB naturally and those that do not. Although, genetic engineering has made transgenic plants to accumulate GB, the levels of GB accumulation in these transgenic plants are relatively low (0.05-5.0 μmol g^{-1} FW; Sakamoto and Murata 2001) when compared with natural accumulators under stress conditions. The highest level of GB was close to 100 μmol g^{-1} FW, in GB-accumulating transgenic plants to date.

Although considerable efforts have been made to increase the overall levels of GB in transgenic plants but reported levels of GB are still relatively low when compared to those in natural accumulators after their exposure to abiotic stress. It has been suggested that the limitation in production of GB in higher plants is either reportedly due to low availability of substrate choline or reduced transport of choline into the chloroplast where GB is naturally synthesized (Huang *et al*. 2000).

Tobacco has been engineered to produce bacterial CDH and COD, as well as plant CMO (Holmstrom *et al*. 2000; Huang *et al*. 2000). Although the engineered genes successfully directed the synthesis of these enzymes, GB accumulated at no more than 0.1 μmol g^{-1} FW in the transgenic plants. The inefficient synthesis of GB in tobacco might have been due to the low-level expression of the transgenes in association with the limited supply of substrate. When exogenous choline was available, levels of accumulated GB increased in transgenic plants that expressed COD or CMO in either the cytosol or the chloroplasts. However, it is important to note that exogenous choline did not increase the net accumulation of GB equally in all transgenic plants. Upon treatment with choline, transgenic *Arabidopsis* that overproduced COD in the cytosol accumulated GB at a concentration approximately 600 μmol g^{-1} DW, whereas tobacco that expressed chloroplast-localized CMO accumulated GB at only 1 μmol g^{-1} FW (Huang *et al*. 2000). Thus, for overproduction of GB in transgenic plants substrate availability must also be considered.

EXOGENOUS APPLICATION OF GLYCINE BETAINE

Due to limitation in production of GB in high quantities in transgenic plants, exogenous application of GB is being successfully used which enhanced internal GB level in numerous low-accumulating or non-accumulating plant species and may help in reducing the adverse effects of abiotic stress and subsequently it can also improve growth and yield (**Table 2**). When GB applied to leaves of plants, it is readily taken up by leaf tissues (Park *et al*. 2006). In the leaves of tomato (*Lycopersicon esculentum*), when GB applied, most of the GB is taken up by the leaves is localized in the cytosol and only a small fractions of the cytosolic GB is translocated to chloroplasts while large amounts of foliar-applied GB were translocated to meristem-containing tissues, including the flower buds and shoot apices (Park *et al*. 2006). It was demonstrated that GB was translocated to actively growing and expanding portions, the long-distance translocation of GB being mediated by the phloem (Makela *et al*. 1996).

Drought and glycine betaine

Water is generally considered the most limiting factor in crop production. Depletion of natural resources such as land and fresh water inevitably accompanies the population explosion; therefore it is crucial that strategies be developed to maintain yields in water-depleted situations. The most common water stress encountered is the water deficit stress known as the drought stress. Drought stress is one of the most adverse factors of plant growth and productivity and considered a severe threat for sustainable crop production in the conditions on changing climate. Drought triggers a wide variety of plant responses, ranging from cellular metabolism to changes in growth rates and crop yields. Accumulation of cellular electrolytes due to the dehydration of protoplasm may also cause disruption of cellular metabolism. Recently, the naturally occurring quaternary ammonium compound, GB has received attention as a compatible solute that may aid in drought tolerance by allowing maintenance of turgor pressure (Agboma *et al*. 1997a). GB also protects physiological processes such as photosynthesis and protein synthesis from the consequences of water deficit.

Crop losses as a consequence of drought stress can be ameliorated by application of osmoprotectants to crop plants. There are various reports demonstrating the positive effect of exogenous GB on plant growth and final crop yield under drought stress in following crops like barley, wheat, soybean, tobacco, maize, sunflower and common beans (Ashraf and Foolad 2007). Exogenously applied GB can rapidly penetrate through leaf surface and be easily transported to other plant organs, where it would contribute to improvement in stress tolerance (Makela *et al*. 1998). Response to drought stress differs noticeably among different crop cultivars due to their inherent differences in drought tolerance (Huang and Zhao 2001). The GB-induced drought tolerance could be attributed to lowering of the osmotic potential due to net solute accumulation in response to water stress, which might help to preserve the metabolic processes, contribute to growth of plants through maintaining turgour in cells (Chimenti *et al*. 2002), and ultimately increase drought tolerance. GB might contribute to restrict cytoplasmic dehydration and maintain leaf turgour in plants subjected to water deficit conditions (Iqbal and Ashraf 2009), thereby maintaining high photosynthetic activities. Thus, increased photosynthetic capacity could then lead to improved capability of the plant to allocate more assimilates to developing seeds (Makela *et al*. 1998). These results are also in agreement with Hussain *et al*. (2009) who reported that exogenous application of GB improved the growth by increasing the water potential, osmotic potential, turgor pressure, water use efficiency and achene yield in sunflower under drought.

Anjum *et al*. (2011) investigated the effect of GB in two

Table 2 Response of exogenous GB application in higher plants enhanced tolerance to various abiotic stresses.

Plant species	Type of stress	Exogenous GB application	Response of exogenous GB application	Reference
Glycine max	Drought (75 and 100 irrigation levels)	3 kg/ha	Increased seeds number	Agboma *et al*. 1997b
Nicotiana tabacum	Drought (50% saturation level for 15 days)	80 mM	Improved water status and increased PSII activity	Ma *et al*. 2007
Helianthus annuus	Drought (20 days after drought)	100mM	Biomass production	Iqbal *et al*. 2008
Triticum aestivum	Drought (60 days after drought)	50 mM	Improved plant biomass	Mahmood *et al.* 2009
Zea mays	Drought (20 days after drought)	100 mM	Enhanced the growth, yield and yield components by maintaining higher antioxidant enzymes activity	Anjum *et al*. 2011
Helianthus annuus	Osmotic stress (-0.6, -1.2 MPa)	25 and 50 mM	• GB did not alleviate the adverse effect of osmotic stress • 50 mM GB increased slight seedling fresh biomass	Iqbal and Ashraf 2006
Lycopersicon esculentum	Salinity (100 mM NaCl)	100 mM	Increased photosynthetic efficiency by decreasing photorespiration	Makela *et al*. 1999
Zea mays	Salinity (10 dS m^{-1})	100 mM	Up-regulated photosynthetic capacity and activities of antioxidant enzymes	Nawaj and Ashraf 2010
Oryza sativa	Salinity (150 mM NaCl)	50 mM	• Enriched proline content • Improved net photosynthetic rate • Improved seed fertility and total grain weight	Cha-um and Kirdmanee 2010
Lolium perenne	Salinity (250 mM)	20 mM	Alleviated cell membrane damage by reducing oxidation of membrane lipid and improved the ion homeostasis	Hu *et al*. 2012
Zea mays	Low temperature (7days chilling period at 4°C)	1 mM at 26°C for 1 day	30% survival rate of suspension cultured cells by preventing lipid peroxidation	Chen *et al*. 2000
Arabidopsis thaliana	Cold acclimation (4.2°C day/night up to 4 weeks)	10 mM	Increased freezing tolerance from -3.1 to -4.5°C	Xing and Rajashekar 2001
Nicotiana tabacum	Cd (100 µM)	10 mM	Intensified the accumulation of GB content and decreased lipid peroxidation and increased CAT activity with reducing Cd accumulation	Islam *et al*. 2009b
Mung bean	NaCl (300 mM)	15 mM	Provide a protective action against salt induced oxidative damage by reducing H_2O_2 and lipid peroxidation level and by enhancing antioxidant defense and methylglyoxal detoxification systems.	Hossain and Fujita 2010
Brassica juncea	Cu (100 mg kg^{-1})	50 mM	Improved growth and biological yield	Priyanka 2011
Mung bean	NaCl (200 mmol L^{-1}, 48 h)	-	Coordinate induction of antioxidant defense and glyoxalase system	Hossain *et al.* 2011
Glycine max	NaCl (EC=11.1 dSm^{-1})	2.5, 5, 7.5 and 10 kg/ha	Exo-GB significantly increased weight of thousands grain (highest; 71% in 10 kg ha^{-1} GB)	Rezaei *et al.* 2012
Zea mays	Drought (30, 50, 70, 100% field capacity)	7.5 kg/ha	• Increased number of branch, number of seed $plant^{-1}$ and 1000-seed weight • Had no significant effect on the plant height	Rezaei *et al.* 2012
	Drought [(60% well watered (750 mL pot^{-1} day^{-1})]	4 kg/ha	GB increased plant height and its strong correlation with leaf area, leaf, stem and ear dry weight	Reddy *et al*. 2013

contrasting cultivars, 'Dongdan-60' and 'ND-95' of maize under drought. The gas exchange and chlorophyll concentration substantially decreased in both cultivars under water stressed conditions. During water deficit conditions, maize cultivars treated with GB, maintained higher gas exchange and chlorophyll synthesis which resulted in improved gas exchange, chlorophyll synthesis, growth and yield of maize. Furthermore, the positive responses to exogenous GB application were more pronounced in 'Dongdan-60' as compared to 'ND-95' in all traits examined under water deficit conditions.

Salinity and glycine betaine

It is estimated worldwide that about one third of the irrigated arable land is already salt affected and that salinization of land is still expanding (Tanji 1990). Salt stress is one of the major environmental factors that restrict plant growth and the productivity of various crop species (Boyer 1982). Salt-induced reduction in plant growth and yield occurs due to various factors such as reduced photosynthetic metabolism, leaf chlorophyll (Chl) content and photosynthetic capacity (Dubey 2005).

In many reports, it has been reported that GB alleviated adverse effect of salt stress by changing photosynthetic activity in many crop plants tomato, maize, wheat and sunflower, which mainly occurred due to stomatal limitations (Ibrahim *et al.* 2006; Raza *et al*. 2006). For instance, Nawaz and Ashraf (2010) noted that foliar application of GB significantly increased the stomatal conductance (g_s) of maize plants under salt stress. Furthermore, net photosynthetic rate (A) and g_s showed a significant positive relationship and similarly, A and g_s were also positively correlated with sub stomatal carbon dioxide. These results indicate that ameliorative effect of GB on net photosynthetic rate in salt-stressed maize plants was mainly due to stomatal limitations.

The reduction in photosynthesis under salt stress can also be attributed to a decrease in chlorophyll content (Delfine *et al*. 1999). Nawaz and Ashraf (2010) found that photosynthetic pigments like chlorophyll *a* or *b* decreased in salt-stressed maize plants. Foliar application of GB increased the leaf chl *b* and total leaf chlorophyll content under saline conditions. Leaf chl *a* was correlated with net photosynthetic rate (A). Such a type of positive relationship between A and chl *a* is in agreement with some previous studies on different crops e.g., alfalfa (Winicov and Seemann 1990), sunflower (Ashraf and Sultana 2000), and wheat (Raza *et al*. 2006). Thus, higher leaf chlorophyll content is one of the additional factors (other than stomatal limitations) that may have contributed to higher photosynthetic capacity of wheat cultivars under saline conditions. This type of relationship between net photosynthetic rate and photosynthetic pigments has also been observed in some trees (Kozlowski 1982). Thus, higher leaf chlorophyll content due to exogenous GB is the other additional factor

that may have contributed to a higher photosynthetic capacity, under saline conditions.

It is evident that most salt tolerant cultivars accumulate more GB than salt sensitive cultivars of same crops, e.g., sugar beet (*Beta vulgaris*), spinach (*Spinacia oleracea*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*) (Raza *et al*. 2006). For instance, Raza *et al*. (2006) found that the moderately salt tolerant cultivar ('S-24' of wheat accumulated more GB than moderately salt sensitive 'MH-97' under salt stress. In addition, exogenous application of GB also improved the photosynthetic capacity and plant water status (osmotic adjustment) of salinized wheat plants, particularly of cv. 'S-24' which could naturally explain the ability of the salt tolerance by 'S-24' than moderately salt sensitive 'MH-97'.

It is well established that salt tolerance is typically characterized by enhanced exclusion of Na^+ and increased absorption of K^+ to maintain optimum K^+/Na^+ ratio in shoots (Gorham *et al*. 1990) GB may have a role in Na^+/K^+ discrimination, which substantially or partially contributes to salt tolerance. For example, Raza *et al*. (2006) noted accumulation of Na^+ in the shoots and roots of both wheat cultivars 'S-24' and 'MH-97' were significantly increased due to salt stress, while K^+ and Ca^{2+} accumulation was decreased. Moreover, application of GB reduced the accumulation of Na^+ accompanied by an increased accumulation of K^+ which resulted in an increased K^+/Na^+ and Ca^{2+}/Na^+, ratios of both cultivars under saline conditions. Overall, cv. 'S-24' was better than 'MH-97' in discriminating Na^+ over K^+ and Ca^{2+}. Thus, it showed GB contributes to salt tolerance via its role in ion homeostasis.

Makela *et al*. (1998) reported that exogenous GB application caused a significant increase in growth and yield in greenhouse and field grown tomatoes. This improvement in growth and/or yield was linked to high endogenous GB level, improved water status of plants (Lopez *et al*. 2002), increased photosynthetic capacity (Yang and Lu 2005). However, adverse effects of exogenous GB on growth of tomato have also been reported (Heuer 2003).

Low temperature and glycine betaine

Each plant has its unique set of temperature requirement which is optimum for its proper growth and development. This temperature may be optimum for one plant and stressful for another. Chilling injury, that causes physical and physiological changes induced by exposure to low temperatures, is another primary factor which limits crop production worldwide. Many species that originated in tropical and sub-tropical regions are susceptible when temperature falls below 15°C (McKersie and Leshem 1994). Many plants accumulate low-molecular-weight compounds with cryoprotectant activity in response to low temperatures (Rajashekar 2000). GB is also known to stabilize membranes during freezing (Zhao *et al*. 1992). The cryoprotective property of GB appears to come from its compatibility with macromolecular structures and functions. It has been suggested that GB can help to stabilize protein tertiary structure and prevent or reverse the disruption of the tertiary structure of proteins caused by non-compatible (perturbing) solutes (Bateman *et al*. 1992). Accumulation of GB in response to low temperatures has been reported in wheat (Naidu *et al*. 1991), barley (Kishitani *et al*. 1994), and strawberry (Rajashekar *et al*. 1999). The accumulation of endogenous GB was closely related to the development of freezing tolerance (Kishitani *et al*. 1994). In addition, significant increase in freezing tolerance has been observed after exogenous application of GB to various plants (Allard *et al*. 1998; Rajashekar *et al*. 1999).

It has been proposed that GB that is supplied exogenously can protect higher plants against stress due to low temperatures (Kishitani *et al*. 1994; Chen *et al*. 2000). Rajashekar *et al*. (1999) reported that exogenous GB was effective in inducing cold tolerance in unhardened and cold-hardening plants of strawberry. The increase in cold tolerance (80%) over untreated controls occurred within 72 h of GB application. Results from the exogenous application of GB to plants showed that GB can induce cold tolerance in leaves, which appear to take up exogenous GB, 24 h after its application (Rajashekar *et al*. 1999). The fact that cold tolerance of unhardened plants was nearly doubled by foliar application of GB which shows the possible involvement of GB on cold tolerance in strawberry plants. This is further supported by the data on time-course accumulation of GB in plants which suggests that GB is involved in the induction of cold tolerance in strawberry leaves. GB has been suggested to provide protection to membranes against freezing in alfalfa (Zhao *et al*. 1992). Sakai and Yoshida (1992) found that GB can increase the freezing resistance of cabbage cells. In addition, it improved freezing survival and regrowth in whole plants. However, in both studies higher levels of GB were used than the other studies.

Xing and Rajashekar (2001) reported that when GB (10 mM) was used as foliar spray the freezing tolerance was found to increase from -3.1 to -4.5°C in *Arabidopsis.* They also showed that *Arabidopsis* plants absorb exogenously applied GB readily and in relatively large quantities. These results show that elevated GB levels in plants can induce freezing tolerance during natural cold acclimation as well as following exogenous application. Improved freezing tolerance in these plants is probably due to its traditional protective role with regard to the function and structure of proteins, enzymes and membranes. Similarly, Coughlan and Heber (1982) observed that GB protected thylakoid membrane against freezing and membrane integrity during freezing in alfalfa and it was proposed that this protection may be due to a weak interaction between the positive quaternary ammonium cation and the anionic carboxyl groups of the exposed membrane proteins. These observations suggest that accumulation of GB may play a role in the induction of freezing tolerance during cold acclimation. However, considering the low concentration of GB in *Arabidopsis thaliana* plants relative to that in typical GB-accumulating species, it is reasonable to suggest that its functions may be much more than just an osmoprotectant or cryoprotectant.

Park *et al*. (2006) reported that exogenous GB application enhanced chilling tolerance in tomato. They found that higher levels of H_2O_2, catalase activity and expression of the catalase gene (*cat1*) immediately after GB application in GB-treated plants suggesting that, in addition to protecting macromolecules and membranes directly, GB-enhanced chilling tolerance may involve the induction of H_2O_2-mediated antioxidant mechanisms, e.g., enhanced catalase expression and catalase activity. Interestingly, large amounts of GB can be found in meristematic tissues, including the shoot apices and flower buds of treated plants and the high levels in these tissues may be critical for plant survival and enhanced recovery of growth after release from chilling temperatures.

Heavy metal and glycine betaine

Heavy metals are important environmental pollutants and many of them are toxic even at very low concentrations. Heavy metals like As, Cd, Co, Cu, Cr, Ni and Zn is phytotoxic either at all concentrations or above a certain threshold level. Heavy metals are present in the environment at concentrations that can be hazardous to the biosphere and are biologically magnified through the food chain. They affect the environment by affecting soil properties like fertility, biomass, crop yields and ultimately human health. Excess production of reactive oxygen species (ROS) due to heavy metals is toxic to plants and cause oxidative damage to cellular constituents (Banu *et al*. 2009) but the plants possess several antioxidant defense systems to protect their cells against ROS. GB besides functioning as osmoprotectants suppresses production of free radicals and ROS. The ascorbate-glutathione (ASC-GSH) cycle is one of crucial mechanisms scavenging ROS in this defense system (Noctor and Foyer 1998).

Islam *et al*. (2009a) found that exogenous application of GB suppressed Cd-induced ROS production by increasing the activities of ASC-GSH cycle enzymes and significantly restored the membrane integrity under Cd stress in tobacco bright yellow-2 cells. Their study also suggests increased GB content caused a decreased lipid peroxidation and increased the activity of SOD and CAT to mitigate the detrimental effects of Cd stress (Islam *et al*. 2009b). GB exhibits similar effects on the activity of ascorbate peroxidase (APX) in detoxifying H_2O_2, generated by Cd stress.

Priyanka (2011) reported that GB (50 mM) counteracted the oxidative stress in Cu-stressed mustard plants by elevating the level of proline and of antioxidant enzymes like CAT, POX, SOD which manifested in terms of improved growth and biological yield.

Oxidative stress and glycine betaine

When plants are growing even under non stress conditions, ROS are regularly generated as metabolic byproducts (Foyer and Harbinson 1994). The ROS such as superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen are produced when electrons from electron transport chain in mitochondria and chloroplasts are leaked and react with molecular O_2 in the absence of other electron acceptors (Mittler 2002). Excessive levels of ROS result in oxidative damage to plants (e.g., nucleic acid, proteins and/or lipids) and causes degradation of chlorophyll pigments (Schutzendubell and Polle 2002). Therefore, ROS generation should remain within plant compatible limits. Under non-stress/ normal conditions these ROS are scavenged by antioxidant defense compounds such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) (Foyer and Nocter 2003) and prevent ROS from reaching toxic levels. All forms of abiotic stresses, including drought, salinity, high temperature, chilling, freezing and heavy metals, cause an oxidative burst in plant cells. An intricate network of defense and repair mechanisms counteracts these oxidation reactions. Any imbalance between ROS generation and safe detoxification represents a metabolic state that is referred as oxidative stress. Therefore, understanding of oxidative stress, antioxidant defense mechanisms and alleviation of oxidative damage are important for protecting plants under stress conditions. The accumulation of GB in plants is very important for protection against oxidative stress, induced by abiotic factors. In addition to their osmoprotective roles, GB also enhances antioxidant defense mechanisms against stress damage (Ma *et al*. 2006; Hoque *et al*. 2007a).

Studies *in vitro* have demonstrated that GB, on its own, does not have antioxidative activity (Smirnoff and Cumbes 1989). It has been proposed that GB functions indirectly via the induction of the synthesis or activation of ROS-defense systems. Such a scenario has been demonstrated both after the exogenous application of GB and in transgenic plants that accumulate GB. Hoque *et al*. (2007b) investigated the effects of exogenously applied GB on levels of antioxidants and on the activities of enzymes in the ASC-GSH cycle in tobacco suspension-cultured cells that were exposed to salt stress. They demonstrated that salt stress significantly depressed the levels of ASC and GSH, as well as the activities of enzymes in the ASC-GSH cycle, such as APX, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). Exogenously applied GB increased the activities of all of these enzymes with the exception of MDHAR. However, GB had no direct positive effects on the activities of enzymes in the ASC-GSH cycle under normal conditions, but only under salt stress. Thus, the GB-enhanced activities of enzymes in the ASC-GSH cycle might protect tobacco cells against salt-induced oxidative stress.

Yang *et al*. (2007) established the relationship between genetically engineered tobacco plants and their ability to accumulate GB. Their results show that the production of ROS was greatly enhanced during heat stress but this production was much less in transgenic plants than in wild type suggesting that the accumulation of GB *in vivo* may alleviate the production of ROS during heat stress. It can be suggested that the improved repair process of PSII induced by the accumulation of GB *in vivo* might be explained by the production of less ROS in transgenic plants during heat stress which was because of the enhanced activities of a series of antioxidant enzymes as well as the contents of ASC and GSH.

MECHANISM AND PROTECTIVE ROLE OF GLYCINE BETAINE

Protection of photosynthetic machinery

The actions of various types of abiotic stress producing ROS inhibit the repair of PSII. It is very likely that CO_2 limitation, low temperature, moderate heat, salinity, and heavy metals generate ROS by suppressing the fixation of CO_2 and decreasing levels of 3-phosphoglyceric acid. The suppression of photosynthetic fixation of CO_2 leads to decline in the level of $NADP^+$. Due to the absence of the major acceptor of electrons ($NADP^+$) in photosystem I (PSI) accelerates the transport of electrons to molecular oxygen with the generation of H_2O_2 via $O_2^{\cdot-}$ production (Asada 1999). These ROS, in turn, inhibit protein synthesis and, thus, the repair of PSII. Thus, ROS increase the extent of photoinhibition by inhibiting the repair of PSII. The photodamage to PSII is characterized by photochemical damage to a constituent of the PSII reaction centre i.e., D1 protein, whereas the repair of the PSII complex involves several steps that ensure the removal and replacement of damaged D1 protein.

Fig. 2 shows that GB enhances the tolerance of the photosynthetic machinery to photoinhibition under various stress conditions by several segmental biochemical processes (a) removal of the photodamaged D1 protein from the PSII complex; (b) transcription of the *psbA* gene in chloroplast, which encodes the precursor to the D1 protein, and translation; (c) insertion of the precursor polypeptide into the PSII complex; (d) removal, by cleavage, of the carboxyl-terminal extension of the precursor to generate the mature D1 protein, and reconstitution of the fully functional PSII complex. The step(s) at which GB acts specifically to promote the restoration of active PSII complexes and the way in which it does this remain to be determined (Aro *et al*. 1993).

In addition, when the photosynthetic fixation of CO_2 is depressed under abiotic stress, excess electrons from PSI are converted to ROS, which inhibit the repair of photodamaged PSII by inhibiting the synthesis of the pre-D1 protein at the translation step. GB might protect the CO_2-fixing enzymes (Rubisco and Rubisco activase) under abiotic stress, thereby sustaining the fixation of CO_2, which, in turn, depresses the production of ROS. Furthermore, GB activates the expression of genes for ROS-scavenging enzymes, which degrade ROS and decrease the levels of ROS in cells, with resultant mitigation of the effects of the abiotic stress on the photosynthetic machinery (Chen and Murata 2011).

Ohnishi and Murata (2006) examined the effects of salt stress and the synthesis of GB on the photoinhibition of PSII under salt stress. Salt stress due to 220 mM NaCl enhanced the photoinhibition of PSII, while GB, which had been synthesized *in vivo*, protected PSII against photoinhibition under these conditions. However, neither salt stress nor the synthesis of GB affected the photodamage to PSII. By contrast, salt stress inhibited the repair of photodamaged PSII and GB reversed this inhibitory effect of salt stress. Pulse-chase labeling experiments revealed that salt stress inhibited the synthesis of the D1 protein *de novo* and the degradation of D1 protein in photodamaged PSII. By contrast, GB protected PSII against inhibition of the degradation and synthesis of the D1 protein under salt stress. Neither salt stress nor GB affected levels of psbA transcripts. These observations suggested that betaine might counteract

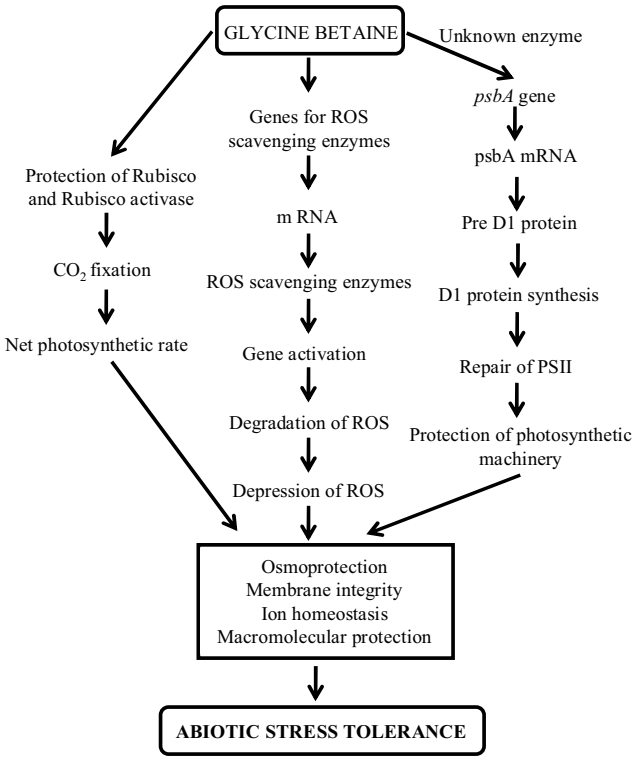

Fig. 2 A hypothetical scheme shows the mechanism and protective roles of glycine betaine directly or indirectly via ROS scavenging system.

the inhibitory effects of salt stress, with resultant accelerated repair of photodamaged PSII.

Moreover, Yang *et al*. (2005) examined the effects of moderate heat stress and the synthesis of GB on photoinhibition in tobacco plants, which had been transformed to synthesize GB *in vivo*. They found that moderate heat stress inhibited Rubisco activase and, as a result, limited the fixation of CO_2. These conditions accelerated the generation of ROS, which, in turn, inhibited the repair of PSII. It is likely that GB protects Rubisco activase against moderate heat induced inhibition. The mechanisms that protect the PSII-repair system against salt stress and mild heat stress seem to have some features in common. Further studies are necessary for a full understanding of the mechanisms responsible for the effects of various kinds of environmental stresses on photoinhibition and photosynthesis.

Protection of transcriptional and translational machinery

The proposed mechanisms for GB-mediated abiotic stress tolerance include stabilization of native structure of proteins and enzymes, osmoregulation, membrane integrity, protection of photosynthesis and detoxification of reactive oxygen radicals produced during stress. However, these mechanisms may not explain observed stress tolerance in transgenic plants completely because of the low levels of accumulation of GB in transgenic plants. The effective concentrations of GB after uptake of exogenously applied GB or as a result of the genetically engineered synthesis of GB *in vivo* are in the milimolar range, as the effective concentrations of plant hormones (Einset *et al*. 2007). Thus, it is also reasonable to postulate that the involvement of GB in the protection of the transcriptional and translational machinery might be mediated through the induction of expression of specific genes under stress conditions whose products are involved in the development of stress tolerance. Rajendrakumar *et al*. (1997) have demonstrated that GB lowers the melting temperature of double stranded DNA *in vitro*. This would make GB a candidate to regulate gene expression by activating replication and transcription in a high salt environment. cDNA microarray analysis revealed the up or downregulation of some endogenous genes in transgenic plants. GB when applied exogenously resulted in change in transcript levels of *WCOR410* and catalase gene in wheat and tomato plants, respectively. Allard *et al*. (1998) examined the accumulation of three cold-responsive proteins (whet cold-regulated 410 (WCOR410), wheat cold-specific 120 (WCS120 and WCS413) after the exogenous application of GB to wheat plants, suggesting that GB has an ability to enhance the transcription *in vivo* of genes that are involved in stress tolerance. Application of exogenous GB in tomato plants resulted in elevated level of the transcript of *cat1* gene and enhanced catalase activity, and this effect was strongest one day after chilling treatment (Park *et al*. 2006). cDNA microarray was also used to compare gene expression in flower buds of wild-type and *cod-A* transgenic tomato, where expression of 30 genes was induced and that of 29 repressed (Park *et al*. 2007). Moreover, Bourot *et al*. (2000) have 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. Thus, GB and functions of their products synthesized in transgenic plants, seems to be capable of activating specific genes might help in understanding of GB-enhanced stress tolerance in plants.

CONCLUSION

Exogenous applications of GB and transgenic approaches have shed some light on the ways in which GB protects plants against abiotic stresses. Current research efforts are focused on the elucidation of the mechanisms by which GB protects the cellular machinery *in vivo* and how, as a result, it enhances the tolerance of whole plants to abiotic stress. The proposed mechanisms for GB-mediated abiotic stress tolerance include osmoprotection, protection of membrane integrity and subcellular structures and ROS detoxification. Given the low levels of accumulation of GB in transgenic plants, these mechanisms may not explain observed stress tolerance in transgenic plants completely. Further identification of GB-inducible genes and the functions of their products could advance our understanding of GB-mediated stress tolerance in plants.

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