Review

Role of Glycine Betaine in the Thermotolerance of Plants

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Abstract: As global warming progresses, agriculture will likely be impacted enormously by the increasing heat stress (HS). Hence, future crops, especially in the southern Mediterranean regions, need thermotolerance to maintain global food security. In this regard, plant scientists are searching for solutions to tackle the yield-declining impacts of HS on crop plants. Glycine betaine (GB) has received considerable attention due to its multiple roles in imparting plant abiotic stress resistance, including to high temperature. Several studies have reported GB as a key osmoprotectant in mediating several plant responses to HS, including growth, protein modifications, photosynthesis, gene expression, and oxidative defense. GB accumulation in plants under HS differs; therefore, engineering genes for GB accumulation in non-accumulating plants is a key strategy for improving HS tolerance. Exogenous application of GB has shown promise for managing HS in plants, suggesting its involvement in protecting plant cells. Even though overexpressing GB in transgenics or exogenously applying it to plants induces tolerance to HS, this phenomenon needs to be unraveled under natural field conditions to design breeding programs and generate highly thermotolerant crops. This review summarizes the current knowledge on GB involvement in plant thermotolerance and discusses knowledge gaps and future research directions for enhancing thermotolerance in economically important crop plants.

Keywords: abiotic stresses; heat stress; high temperature; osmolytes; heat tolerance; transgenic plants

1. Introduction

Temperature can adversely affect the normal functioning of plant metabolism [1,2]. In the last few decades, climate change-induced rising temperatures have beome a major challenge for modern crop production, especially in southern Mediterranean regions [3]. Thus, efforts to achieve maximum crop yields to ensure food security for an ever-increasing human population remain challenging. Global food security could be jeopardized if mitigation efforts are not implemented aggressively [4]. Any fluctuation in an environmental cue such as temperature can directly affect the production of temperature-sensitive crops, and hence food security, causing considerable losses to growers and other stakeholders. According to the Inter-Governmental Panel on Climate Change, global temperatures increased by 0.74 °C within a century (1906–2005), which was ascribed to ongoing anthropogenic activities and their resultant greenhouse gases [5]. The terrestrial surface temperature is expected to increase by a further 1–6 °C by 2050, with arable areas projected to see the greatest increases [6]. There is little doubt that the ongoing climate change will affect all societies inhabiting the globe [7,8], decreasing crop production, and threatening food security. Therefore, strategies are needed to ameliorate its effects on modern agriculture.

Heat stress is a condition of unfavorable temperature causing irreversible damage to plants [9]. High-temperature stress adversely affects plant physio-biochemical and molecular characteristics, resulting in poor plant growth and development [10]. At the physiological level, heat stress negatively influences photosynthesis by adversely affecting

the oxygen evolving complex, photosystem II, RuBisCo, and energy-(ATP) producing processes [11,12]. Furthermore, heat stress-induced disturbance of the electron transport chain leads to excessive reactive oxygen species (ROS) production in different cell organelles, such as mitochondria and chloroplasts, causing severe damage to DNA and cell membranes by inducing lipid peroxidation and ultimately causing cell death 13]. Increasing the capacity of heat stress-induced excessive ROS scavenging is considered an efficient defense strategy for ameliorating heat stress in plants [13]. A plant's thermotolerance ability is attributed to enhanced plasma membrane thermostability and reduced toxic ROS levels [14]. Plants have naturally adapted various defense mechanisms to counteract harsh environmental conditions such as heat stress. These defense mechanisms include an antioxidant machinery, osmolyte accumulation, maintenance of membrane integrity, and increased biosynthesis of heat-shock proteins (HSPs) by upregulating their associated genes' expression [15,16]. These defense mechanisms are involved in cellular defense against heat stress. Osmolyte accumulation has a significant role in mediating stress tolerance in plants (reviewed in Zulfiqar et al. [17]). Various studies have reported enhanced GB accumulation in plants under heat stress, revealing the positive role of this osmolyte in heat stress tolerance [18–22]. Sorwong and Sakhonwasee [23] stated that exogenous GB application alleviated the heat stress-induced reduction in leaf gas exchange traits.

There are no critical reviews on the role of GB in heat stress tolerance. Here, we summarize the fundamental impact of GB in inducing heat stress tolerance in economically important crops.

2. GB Structure and Biosynthesis in Plants

Glycinebetaine is an *N,N,N*-trimethylglycine quaternary ammonium compound that is naturally produced in numerous living organisms, including plants [24]. At physiological pH, GB is electrically a neutral molecule, but dipolar in nature. There are two pathways related to GB biosynthesis in plants [25]. The initiating metabolites for these pathways are choline and glycine [26]. GB biosynthesis in plants occurs via N methylation of glycine or the oxidation of choline [27]. GB biosynthesis, particularly in higher plants, is a two-step pathway beginning with choline, which is catalyzed by a ferredoxin-dependent Rieske-type protein, namely, choline monooxygenase (CMO), and by a soluble NAD+-dependent enzyme [28,29]. Betaine aldehyde is oxidized by NAD$^+$-dependent betaine aldehyde dehydrogenase (*BADH*) to produce GB. Both *BADH* and CMO generally exist in chloroplast stroma [24,30] (Figure 1).
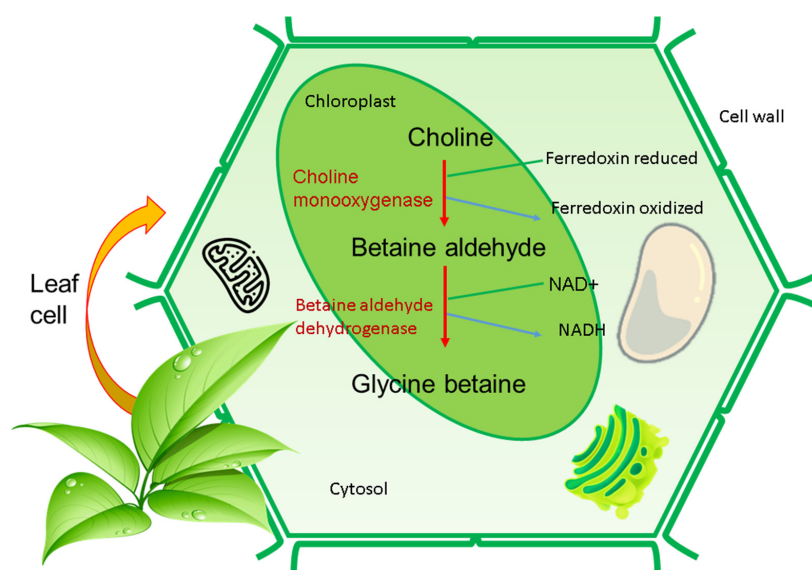
Figure 1. Biosynthesis of GB in plant cells (Figure adapted from Sakamoto and Murata [24]; Ashraf and Foolad [31]).

3. Glycine Betaine-Accumulating and -Non-Accumulating Plants

Glycine betaine is a vital compatible osmolyte that plays multifarious roles in plant growth and metabolism. However, plant species differ in naturally accumulating GB. It is now evident that GB synthesis occurs in both chloroplasts and cytosol [32,33], but chloroplastic GB has been positively related to stress tolerance, whereas cytosolic GB has not shown such a relationship [32]. Thus, the presence of high amounts of GB in a plant may not necessarily account for its enhanced stress tolerance. Metabolic restriction of GB synthesis in plants has been ascribed to the availability of the substrate (choline) [33]. Since choline occurs in the cytosol [34], its transport to chloroplasts through appropriate transporters is essential for GB synthesis [34,35]. GB-non-accumulating plants have either limited amounts of intrinsic choline or poor activity of choline transporters in the chloroplast envelope [35]. Thus, genetic engineers need this information to generate transgenic lines with high GB-accumulating ability.

Plants accumulate various amounts of GB; naturally accumulating plants under normal and stress conditions are categorized as GB accumulators, while non-accumulating plants do not increase GB level under stress [36]. Table 1 lists GB accumulators and non-accumulators. Natural GB accumulators accrue a certain amount of GB solely under stressful cues [18,21]. For example, Alhaithloul et al. [22] studied the responses of *Catharanthus roseus* and *Mentha piperita* under HS and drought stress, individually and in combination. They reported that the level of GB increased by 46% and 58% for *C. roseous* and *M. piperita*, respectively, in response to HS, and more so under combined heat and drought stress. This evolutionary adaptation enables plants to survive under a varied range of climatic conditions. Screening plants for their ability to accumulate osmolytes, particularly GB, offers a way to target such plants for acquiring GB biosynthesis-related genes to develop GB-producing plants.

**Table 1.** Glycine betaine accumulating crops.

GB Accumulators	Accumulating Condition	References
Plant families with known naturally high accumulation of GB: Asteraceae, Chenopodiaceae, Poaceae, and Solanaceae	Different types of stresses	[37–39]
Spinach (*Spinacia oleracea*)	Naturally accumulates under non-stress conditions; GB levels increase under stress conditions	[31,32,36]
Sugar beet (*Beta vulgaris*)	Naturally accumulates under non-stress conditions; GB levels increase under stress conditions	[31,32,36]
Barley (*Hordeum vulgare*)	Naturally accumulates under non-stress conditions; GB levels increase under stress conditions	[31,36,40]
Wheat (*Triticum aestivum*)	Naturally accumulates under non-stress conditions; GB levels increase under stress conditions	[31,36,40]
Sorghum (*Sorghum bicolor*)	Naturally accumulates under non-stress conditions; GB levels increase under stress conditions	[41,42]
Maize (*Zea mays*)	Naturally accumulates under non-stress conditions; GB levels increase under stress conditons	[36,43–45]
GB-non-accumulators		
Rice (*Oryza sativa*)	Non-stress and stress conditions	[31,36,46,47]
Mustard (*Brassica* spp.)	Non-stress and stress conditions	[46]
Arabidopsis (*Arabidopsis thaliana*)	Non-stress and stress conditions	[33,36,46]
Tobacco (*Nicotiana tabacum*)	Non-stress and stress conditions	[33,46]
Tomato (*Solanum lycopersicum*)	Non-stress and stress conditions	[33,36,46,48]
Potato (*Solanum tuberosum*)	Non-stress and stress conditions	[36,46]

4. Mechanisms of GB-Mediated Thermotolerance

Glycine betaine is a compatible osmolyte that likely plays an important role in osmoregulation in plants subjected to extreme environmental cues, including high-temperature stress) [21,49]. Additionally, it is likely that GB activates signaling molecules such as

calcium-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs) [50], which could activate stress-responsive and heat-shock transcription factor (HSF) genes [51,52]. The activated stress-responsive genes may boost the natural defense system by enhancing the activities of enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), which may alleviate the negative impact of uncontrolled ROS causing oxidative damage triggered by heat stress)[53] (Figure 1). The elimination/reduction of ROS may keep biological membranes intact [54]. Furthermore, activated HSF genes may lead to the synthesis and activation of HSPs [55]. Most HSPs can also act as chaperones, which can prevent heat-induced aggregation of proteins [56]. The role of HSPs in plant thermotolerance has been elucidated in several comprehensive reviews [56,57]. GB can also significantly prevent photoinhibition by stabilizing the structure of the O_2-evolving center (PSII) [19,58]. Thus, overall, GB can stabilize photosynthesis in heat-stressed plants, promoting growth under heat stress.

It is now evident that high temperatures cause many metabolic changes in plants that involve intricate reprogramming of cellular activities to safeguard organellar ultrastructures and functions under heat stress [59]. Although some promising roles of GB are depicted in Figure 1 for counteracting heat-induced physiological disorders, intensive research is needed to elucidate how and to what extent GB can regulate some key processes involved in plant thermotolerance, other than those highlighted in Figure 2. For example, very little information is available on the crosstalk between GB and other biomolecules, including various plant growth regulators.
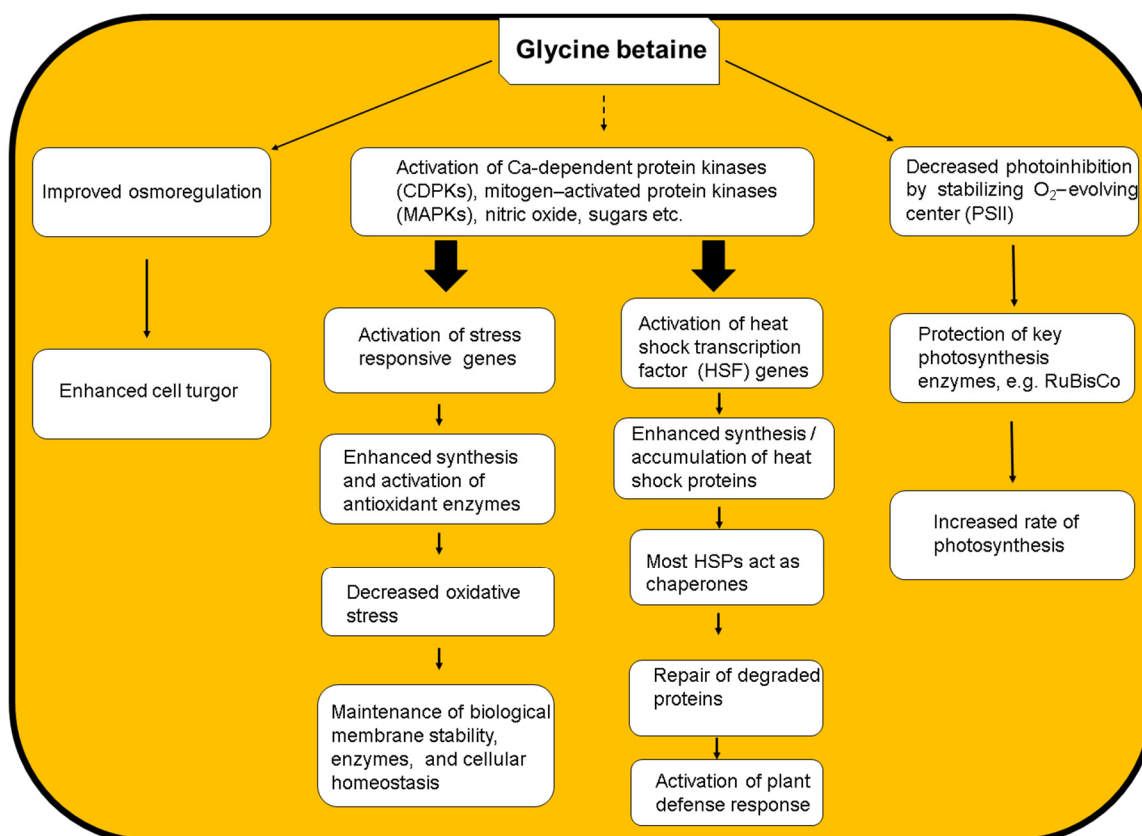
Figure 2. Proposed mechanism of glycine betaine-mediated thermotolerance in plants.

5. Improvement in Heat Tolerance through Exogenous Application of GB

Exogenous application of GB improves thermotolerance in many plants by enhancing their growth and yield via counteracting metabolic maladjustments caused by HS (Table 2). For example, while appraising the role of exogenous GB application on heat-stressed tomato plants, Li et al. [60] reported enhanced seed germination, expression of heat-

shock genes, and accumulation of heat-shock protein 70 (HSP70). Likewise, exogenous GB supplementation likely controls many other key metabolic processes in heat-stressed plants. For example, exogenously applied GB protected photosystem II (PSII) in heat-stressed plants of *Hordeum vulgare* [61] and *Triticum aestivum* [20] and decreased the relative membrane permeability and leakage of ions such as Ca^{2+}, K^+, and NO_3^- in heat-stressed barley seedlings [62]. Furthermore, GB supplied to sprouting sugarcane nodal buds under HS markedly reduced H_2O_2 generation, increased K^+ and Ca^{2+} contents, and increased the levels of endogenous GB, free proline, and soluble sugars, enhancing the overall growth [63]. Sorwong and Sakhonwasee [23] stated that exogenous GB supplementation alleviated the heat stress-induced reduction in CO_2 assimilation rate, stomatal conductance, relative water content, and transpiration rate in marigold. The high-temperature-induced oxidative stress in marigold was mitigated due to reduced levels of H_2O_2, peroxide, superoxide, and lipid peroxidation [23]. Exogenous application of GB during mid-flowering in heat-stressed tomato in the field increased fruit yield [64]. In apple, the application of GB enhanced photosynthesis under individual HS or drought stress and combined stresses [65]. In a three-year field study, Chowdhury et al. [66] evaluated the role of GB and potassium nitrate in heat-stressed late-sown wheat; these osmolytes improved grain yield under heat stress compared to the control. Hence, it is clear that exogenously applied GB mediates HS. However, future studies should focus on field-based heat stress evaluations of different crops.

Table 2. Effect of exogenously applied GB on the regulation of different physio-biochemical attributes in heat-stressed plants.

Crop	Heat Stress Range	GB Concentration Applied	Exogenously Applied GB-Induced Regulation of Different Attributes in Heat-Stressed Plants	Reference
Tomato (*Lycopersicon esculentum*)	34 °C	0, 0.1, 1, and 5 mM GB	• Improved seed germination • Enhanced expression of heat-shock genes and accumulation of HSPs	[60]
Barley (*Hordeum vulgare*)	45 °C	10 mM	• Protective effect on oxygen-evolving complex by increasing connectivity among PSII antennae, inducing greater PSII stability	[61]
Wheat (*Triticum aestivum*)	25/20 °C day/night	100 mM	• Maintenance of higher chlorophyll content, PSII photochemical activity, and net photosynthetic rate • Reversed the decline in *psbA* gene transcription • Accelerated endogenous accumulation of GB in leaves • Decreased photoinhibition by D1 protein synthesis	[20]
Wheat (*T. aestivum*)	30–38 °C	100 and 50 mM	• Improved yield • Marginally improved the relative membrane permeability	[66]
Barley (*H. vulgare*)	40/32 °C day/night	10, 20, 30, 40, and 50 mM	• Improved growth, photosynthesis, and water relations • Decrease ion leakage	[62]

Table 2. *Cont.*

Crop	Heat Stress Range	GB Concentration Applied	Exogenously Applied GB-Induced Regulation of Different Attributes in Heat-Stressed Plants	Reference
Sugarcane (*Saccharum* spp.)	42 °C	20 mM	• Improved bud sprouting • Decreased H_2O_2 production • Improved soluble sugar accumulation • Improved K^+ and Ca^{2+} content • Enhanced the endogenous levels of osmolytes	[63]
Marigold (*Tagetes erecta*)	39/29 °C day/night	0.5 and 1 mM	• Improved leaf gas exchange traits • Decreased ROS accumulation	[23]

Abbreviations: HSPs: Heat-shock proteins, PSII: Photosystem II, ROS: Reactive oxygen species, *psbA*: PSII protein D1 precursor gene.

6. Genetic Engineering for Enhanced Thermotolerance

Developing transgenic plants for thermotolerance is a cost-effective and efficient biotechnological approach for achieving optimum agricultural production under the changing climate scenario [17]. Genes involved in encoding GB biosynthetic enzymes in different organisms and plants have been cloned to produce transgenic plants overexpressing one or more of these genes to enhance endogenous GB production, improving HS tolerance [67,68] (Table 3). For example, Zhang et al. [68] compared the thermotolerance ability of two transgenic tomato lines containing the betaine aldehyde dehydrogenase (*BADH*) and choline oxidase (*COD*) genes responsible for GB synthesis. They observed that *codA* transgenic plants had higher GB levels, CO_2 assimilation rate, and photosystem II (PSII) photochemical activity and lower accumulation of H_2O_2, $O_2^{\bullet-}$, and malondialdehyde (MDA) than wild-type (WT) plants. In addition, the codA transgenic line had higher heat-response gene expression, heat-shock protein 70 (HSP70) accumulation, and expression of a mitochondrial small heat-shock protein (MT-sHSP), heat-shock cognate 70 (HSC70), and heat-shock protein 70 (HSP70) than WT plants under HS. In another study, Yang et al. [69] reported GB accumulation in tobacco by introducing the *BADH* gene in tobacco, which increased tolerance to high-temperature stress and improved photosynthesis. While transferring the *BADH* gene from spinach to tomato, Li et al. [67] reported enhanced accumulation of GB, antioxidant activity, and photosynthetic capacity by improving D1 protein content, which could repair heat stress-induced damage to PSII. Furthermore, transgenic tomato accumulated less MDA and ROS (H_2O_2 and $O_2^{\bullet-}$), reducing oxidative stress relative to the WT. Reduced oxidative stress and restored PSII from HS-induced enhanced photoinhibition occurred in transgenic tobacco plants transformed with the *BADH* gene relative to the WT [70]. The role of *BADH* in xerophyllic *Ammopiptanthus nanus* under severe stress was confirmed by transferring the *BADH* gene of this plant into *E. coli* treated with 700 mM NaCl at 55 °C; the transgenic bacteria showed tolerance to these combined stresses [71]. The above studies reveal the positive role of GB-related genes in providing stress tolerance in plants. The introgression of GB synthesis-related genes could enhance endogenous GB accumulation to protect plants from heat-induced oxidative stress.

Numerous studies have been conducted on engineering GB biosynthesis [33]. Performance of single-gene-based transgenics under field conditions may not be the same as that reported under controlled or semi-controlled conditions. Thus, the development of transgenic lines by transforming with multiple genes (pyramiding of genes) for enhanced GB biosynthesis under heat stress is plausible.

Table 3. Genetic engineering for enhanced GB accumulation and improved thermotolerance in different crops.

Gene Transformed	Donor/Source	Gene Action	Transgenic Plant Species	Stress Condition	Transgenic Plant Response	References
BADH and *codA*	Spinach (*Spinacia oleracea* L.) as a donor of the *BADH* gene; binary vector pCG/codA for chloroplast-targeted expression of the *codA* gene	Genes related to key enzymes involved in GB synthesis	Tomato (*Solanum lycopersicum*)	Two months after transplanting, plants were exposed to 42 °C for 0–8 h in a growth chamber	• Enhanced GB levels in leaves • Improved CO_2 assimilation and photosystem II (PSII) photochemical activity • Transgenic plants, especially those containing *codA*, accumulated low ROS levels and thus exhibited reduced oxidative stress • Enhanced expression of heat-response genes and accumulation of heat-shock protein 70 (HSP70) • Enhanced thermotolerance	[68]
BADH	Spinach (*S. oleracea* L.)	Gene for betaine aldehyde dehydrogenase	Tobacco (*Nicotiana tabacum*)	Two-month-old seedlings subjected to various temperatures (25–50 °C) for 4 h in a growth chamber	• Increased GB accumulation in vivo increased PSII tolerance under heat stress • Enhanced GB accumulation in vivo improved the thermostability of PSII reaction centers • Reversed heat-induced PSII photoinhibition • Accumulated low levels of ROS, improving oxidative defense system	[70]
BADH	Spinach (*S. oleracea* L.)	Gene for betaine aldehyde dehydrogenase	Tomato (*S. lycopersicum*)	Six-week-old seedlings placed in a growth chamber at 42 °C for 0, 2, 4, or 6 h	• Higher GB accumulation • Enhanced chlorophyll fluorescence • Increased tolerance to heat-induced photoinhibition • Improved D1 protein content • Enhanced antioxidant enzyme activities • Reduced oxidative stress	[67]

Table 3. *Cont.*

Gene Transformed	Donor/Source	Gene Action	Transgenic Plant Species	Stress Condition	Transgenic Plant Response	References
BADH	Spinach (*S. oleracea* L.)	—do—	Tomato (*Solanum lycopersicum*)	Two-month-old plants exposed to varying temperature regimes (25–45 °C) for 2 h	• Enhanced CO_2 assimilation • Enhanced photosynthesis related to RuBisCo activase-mediated activation of RuBisCo • Enhanced thermotolerance	[69]
BADH	Garden orache (*Atriplex hortensis* L.)	–do–	Wheat (*Triticum aestivum*)	Individual and combined heat stress (40 °C) and drought stress as PEG-6000 (osmotic potential about −1.88 MPa) for 3 h in an artificial chamber	• Improved photosynthesis • Improved antioxidant activities/levels • Improved water status	[72]
codA	Spinach (*S. oleracea* L.)	Gene encodescholine oxidase (COD), a key enzyme for GB synthesis	Rice (*Oryza sativa*)	Plants grown at 28/13 °C for 5 weeks	• Enhanced endogenous GB accumulation • Improved heat and salt stress tolerance	[47]

7. Conclusions and Prospects

Under the changing climate threat, strategies are needed to alleviate the adverse impacts of harsh environmental stresses such as HS on plants. The most expedient strategy is to use the plant's natural defense system for withstanding these stresses. Under HS, many plants naturally accumulate GB, a compound known to mediate HS tolerance via osmoregulation, photosynthetic mechanisms, and signaling molecules, such as CDPKs, MAPKs, nitric oxide (NO), and sugars, which activate stress-responsive genes and HSF genes.

As discussed above, GB biosynthesis has different roles in different organelles; for example, chloroplastic GB is actively involved in stress tolerance, while cytosolic GB lacks such functionality. As a result, high levels of GB in plants are not the only factor enhancing stress tolerance. The principal substrate for GB synthesis is choline, which occurs in the cytosol. Choline transport to the chloroplast takes place via choline transporters. Several problems occur in GB-non-accumulating plants: (1) a limited amount of endogenous choline exists and (2) choline transporters present on the chloroplast membrane do not transport the required level of choline to chloroplasts. Thus, molecular biologists require this information for different crops to develop transgenic lines/cultivars with enhanced GB-accumulating ability.

Plants that do not naturally accumulate GB under HS have less HS tolerance than those that do. Various strategies have been used to increase GB accumulation in these plants to improve their tolerance against HS. Exogenous supplementation of GB in different forms, such as seed priming or plant priming, has enhanced endogenous GB levels and thus improved plant growth and development under HS. Genetic engineering could be used to introduce biosynthetic pathway-related genes into GB-deficient species from plants or microorganisms. While various studies have demonstrated the importance of GB in thermotolerance, very few have revealed the molecular roles of GB in thermotolerance. Moreover, transgenic lines generated for different crops have been based on a single gene transformation, with marked progress in terms of enhanced GB accumulation coupled with improved thermotolerance. However, all these studies have been undertaken under semi-controlled or controlled conditions, and the developed transgenic lines have not been tested under natural field conditions. Thus, further research is needed to generate thermotolerant lines/cultivars for different crops threatened by the rising temperatures of climate change. The effectiveness of exogenous GB application should be tested at the field level.

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