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Roles of Endogenous Glycinebetaine in Plant Abiotic Stress Responses

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1 Introduction

Abiotic stresses, the most common of which are water deficit (Boyer 1982) followed by water logging, high and low temperature, and salinity, annually restrict not only plant growth but also global crop yield. It has been estimated that during the period 1961–2014, drought and heat spells caused a global production loss of US$ 237 billion (Mehrabi and Ramankutty 2017). According to an IPCC report in 2017, occurrences and damages caused by weather extremes will increase in the future due to climate change. The impact of global warming differs regionally, and it is envisaged that developing countries will be affected to a greater extent, resulting in increased food insecurity (Rosenzweig and Parry 1994). Changes in ambient temperature occur more rapidly than changes in stress factors such as water deficit and salinity. Furthermore, temperature extremes aggravate the adverse effects of other stresses, including water deficit and salinity, on crop production and quality. For example, heat stress adversely affects grain quality and final crop yield in 40% of the global irrigated wheat growing area (Fischer and Byerlee 1991). Cold stress, although seasonal, has some similarities to water deficit. As water freezes, it creates concentrated solutions of solutes, thereby subjecting plants to a shortage of liquid water (Sakai and Larcher 1987).

Global agricultural land area is approximately 4.86 billion ha (FAO 2019). It is estimated that less than 10% of the world's agricultural land may be free of major environmental stresses (Dudal 1976). As much as 45% of agricultural land is subject to different kinds of water deficit, and 38% of the world's human population resides

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in those areas (Bot et al. 2000). In relation, the proportion of irrigated field area is approximately 20%, concentrating mostly in Asia (271 Mha) (FAO 2019). In 2015, approximately 510 Mha of total land area, and 19.5% of irrigated agricultural land, was considered saline (FAO and ITPS 2015). Each year a further 2 million ha (about 1%) of the world's agricultural land deteriorates due to salinity, leading to reduced or no crop productivity (reviewed in Ashraf and Foolad 2007). Apart from irrigation, other major contributors to the increasing area of saline soils are poor management practices, low precipitation, high surface evaporation, and weathering of native rocks. However, secondary salinization causes further problems as productive agricultural land is becoming unsuitable for cultivation due to low quality of irrigation water (Munns 2010).

To minimize the effects of abiotic stresses on crop yield, solutions have been actively sought and investigated. These include improving crop tolerance by means of crop management – for example, by the utilization of exogenous and endogenous compounds, including GB – as well as by traditional and molecular plant breeding. Many of the traits resulting in increased abiotic stress tolerance are an interplay of several genes, which make them difficult to modify via traditional and modern plant breeding. Moreover, different abiotic stress factors may provoke osmotic stress, oxidative stress, and protein denaturation in plants. These lead to similar cellular adaptive responses in plants, such as accumulation of compatible solutes, induction of stress proteins, and acceleration of reactive oxygen species (ROS)-scavenging systems (Zhu 2002). Further complexity is associated with phenology as well as species- and cultivar-specific responses to abiotic stresses.

Exposure to a single abiotic stress factor can lead to plants obtaining tolerance against a wide range of future abiotic stress events, which is referred to as priming, acclimation, conditioning, hardening, or cross-stress tolerance (Li and Gong 2011; Walter et al. 2013; Antoniou et al. 2016). This involves a memory phase that separates the primary stress event from the following stress events (Bäurle 2016). During the primary stress phase, changes take place at the physiological, biochemical, molecular, and epigenetic levels. These changes can be transient or maintained throughout the lifetime of a plant and, in some cases, can even be inherited by subsequent generations, for example, in seeds (Mauch-Mani et al. 2017).

Over the last 10 years, significant steps have been taken in understanding the biology of osmolytes and especially GB in plants. New associations and insights between GB, genes, and ROS and plant hormones, for example, have been discovered. This chapter provides an update on the most recent research related to osmolytes with special emphasis on endogenous GB and on the transgenesis approach for GB.

2 Osmoprotectants in Plants Under Stress Conditions

Identifying the mechanisms involved in plant adaptation to multiple abiotic stresses such as drought, salinity, nutrient imbalances, extreme temperatures, and light is essential for breeding new crop varieties. In addition, understanding the role of

factors resulting in increased plant abiotic stress tolerance may assist in developing novel management practices. In this respect, the early dispersion of stress signals, the successive activation of stress-responsive pathways, and finally the responses of plant yield formation are of primary interest to plant biologists, breeders, and agronomists.

Within the last few years, several comprehensive reviews on plant stress and the roles of osmoprotectants in improving plant stress tolerance have been published (Singh et al. 2015; Verma et al. 2016; Zhu 2016; Hossain et al. 2018). Here we summarize the increasing amount of literature on osmoprotection in relation to plant stress tolerance.

In response to different stresses, plants have developed several mechanisms that involve changes at the morphological, physiological, and molecular level. The sensing of various stresses initiates several complex signaling pathways in plants (Hossain et al. 2018 and cited literature). At first, plants recognize the external stress by using multiple sensors present in the plasma membrane or cell wall. Early signaling events usually include changes to intracellular calcium (Ca^{2+}) concentration followed by an increase in secondary messengers, like reactive nitrogen species (such as nitric oxide), ROS (such as hydrogen peroxide), reactive carbonyl species (such as methylglyoxal), cytosolic calcium ions (Ca^{2+}), hydrogen sulfide, and kinases.

In addition, groups of plant hormones (auxins, gibberellins, cytokinins, abscisic acid, ethylene, salicylic acid, jasmonates, brassinosteroids, and strigolactones) participate in plant defense responses (Kurepin et al. 2015; Verma et al. 2016; Xu et al. 2018). Their signaling pathways are interconnected to assist the generation of an efficient stress response. Currently, the fundamental molecules in plant cells and tissues for the acquisition of stress tolerance are considered to be plant hormones. The compounds collaborate with each other to regulate gene expression, resulting in the modification of membrane rigidity and fluidity, changes in the levels of ROS and methylglyoxal detoxifying enzymatic and nonenzymatic antioxidants, and an increase in the synthesis of osmolytes and stress-related proteins. The complex set of responses at the cellular level is also considered to lead to the cross-stress tolerance discussed recently by Hossain et al. (2018).

To improve plant tolerance to abiotic stresses such as excess light, water deficit, extreme environmental temperatures, or salinity, the osmotic potential of plant cells must increase. This occurs by the enhancement of cell solutes (reviewed in Singh et al. 2015, Stadmiller et al. 2017), which can be inorganic or organic. In general, inorganic solutes are energetically less expensive but may interfere with metabolism. Organic solutes are energetically more expensive but usually have only minor or no effect on metabolism. In addition, salts in the soil negatively affect water absorption by roots and may result in ion toxicity due to the accumulation of sodium (Na^+) and chloride (Cl^-) ions in the plant. Under stress conditions, a significant enhancement of extracellular salt concentration results in water efflux, which decreases cell volume and increases the concentration of macromolecules inside the cytoplasm. Accordingly, an increase of common solutes alone, such as organic acids and inorganic ions, may lead to ionic and nutritional imbalance and may prevent the activity of important plant enzymes. Therefore, the localization of common solutes

is mainly in the vacuoles, where their increased concentration does not lower the metabolic activity of the cell.

In contrast to common solutes, plants can produce different types of compatible organic solutes in response to various stresses (Burg and Ferraris 2008, Singh et al. 2015 and cited literature). In many cases, these solutes seem to accumulate in low concentrations when considered from the whole-plant perspective. However, they typically accumulate in the cytoplasm with high concentrations and do not adversely affect metabolic activity in the cell. Compatible solutes are highly soluble compounds, usually nontoxic at high cellular concentrations, and typically have low molecular weight.

Compatible solutes protect plant cells and tissues from stress through several ways. These include contributing to cellular osmotic adjustment, protecting membrane integrity, stabilizing enzymes and proteins, and the detoxification of ROS (Burg and Ferraris 2008 and cited literature, Stadmiller et al. 2017, Hossain et al. 2018 and cited literature). Some compatible solutes can also act as antioxidants. Moreover, they may play a role in stress tolerance by regulating gene replication and transcription (reviewed in Giri 2011 and Hossain et al. 2018). Because some compatible solutes also protect cellular components from dehydration injury, they are called osmoprotectants.

Recently, Singh et al. (2015) categorized osmoprotectants into three different groups: osmoprotectants containing ammonium compounds (polyamines, GB, β-alanine betaine, dimethylsulfonio propionate, and choline-O-sulfate), osmoprotectants containing sugars and sugar alcohols (trehalose, fructan, mannitol, D-ononitol, and sorbitol), and osmoprotectants containing amino acids (proline and ectoine). The specific role of different osmoprotectants in plant metabolism and stress tolerance has recently been reviewed by Singh et al. (2015) and Hossain et al. (2018). The majority of osmoprotectants avoid participation in biochemical reactions and are stored in the cytosol.

In addition to the conventional osmoprotective role of the compatible solutes, osmoprotectants also detoxify the adverse impacts of stress (e.g., from salinity, water deficit, and cold stress) through two different mechanisms. The first mechanism improves the antioxidant defense system, whereas the second one improves the sustainability of ion homeostasis (reviewed in Singh et al. 2015).

In terms of the antioxidant defense system, several studies (Singh et al. 2015; Hossain et al. 2018; Wei et al. 2017; Razavi et al. 2018; Rady et al. 2018) have indicated that under various stress circumstances, osmoprotectants such as polyamines, GB, sugar alcohols, and proline upregulate antioxidant enzyme activities and increase the concentration of nonenzymatic antioxidants to reduce the adverse effects of oxidative stress. Well-known antioxidant enzymes include superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase and some other nonenzymatic low-molecular-weight antioxidants, like glutathione, ascorbate, and carotenoids. Both enzymes and antioxidants have the capability of providing protection via reducing the toxicity of ROS. In a series of detoxifying mechanisms, plants enhance the production of the metalloenzyme superoxide dismutase, which is responsible for the conversion of superoxide to hydrogen peroxide. The breakdown

of hydrogen peroxide is then catalyzed by CAT and peroxidases. The modulation of the glyoxalase (Gly 1 and Gly 2) and antioxidant defense systems by heat, cold, or osmo-priming has also shown the importance of osmoprotectants for induced cross-stress tolerance. Accordingly, osmoprotectants are promising compounds for improving crop abiotic stress tolerance through the enhancement of the antioxidant system.

During stress caused by salinity and water deficit, the sustainability of ion homeostasis is affected by the accumulation of osmoprotectants providing osmotic adjustment via specific ion exchange activity (Singh et al. 2015 and cited literature, Wei et al. 2017). Under salinity stress, the most common effect is a reduction of plant growth due to specific ion toxicity, such as from Na^+ and Cl^-. This also reduces the uptake of essential nutrients like phosphorus (P), potassium (K^+), nitrogen (N), and calcium (Ca). The toxic ions negatively impact intracellular K^+ influx, reducing the uptake of K^+ by cells. Some osmoprotectants may maintain low cytoplasmic Na^+ concentration in the cell by decreasing K^+ efflux and increasing Na^+ efflux, resulting in an optimal K^+/Na^+ ratio. In addition, osmoprotectants may increase efflux of Na^+ from the roots to the environment, leading to less Na^+ transfer to plant leaves. Thus, it has been proposed that some osmoprotectants also regulate ion channels and transporters in plants (Wei et al. 2017).

3 Endogenous Glycinebetaine and Plant Abiotic Stress Responses

GB is usually classified as an osmolyte, an osmoprotectant, and a compatible solute. GB could also be regarded as a biostimulant, i.e., a non-fertilizer compound applied in low concentrations that promotes either plant growth, abiotic stress tolerance, or crop quality. Osmolytes and osmoprotectants have gained increased attention over the last two decades. A search in Google Scholar for articles related to GB found 338 published before 1979 and 25,800 published in the decade up to February 2019 (Fig. 1).

GB (2-N,N,N-trimethylammonio acetate or N,N′,N″-trimethylglycine), earlier known as lycine or oxyneurine, is a quaternary amine derived from glycine with an average molecular mass of 117.15 (Fig. 2). Due to its zwitterionic nature, it is highly soluble and has low viscosity (Yancey et al. 1982; Yancey 2005). GB is a nontoxic, colorless, tasteless, and odorless compound that accumulates in many plant species, especially in halophytes, when grown under abiotic stresses (see comprehensive list of plant species available in Paleg and Aspinall (1981)).

In higher plants, GB is synthesized as a result of the two-step oxidation of choline (Cromwell and Rennie 1954). The first step is catalyzed by choline monooxygenase (CMO), and the second step is mediated by betaine aldehyde dehydrogenase (BADH). The gene expression of CMO and BADH is induced by salinity, water deficit, and temperature stresses in various organisms (for a review, see Hashemi et al. (2018)). Under osmotic stress, changes of turgor may initiate the signal trans-

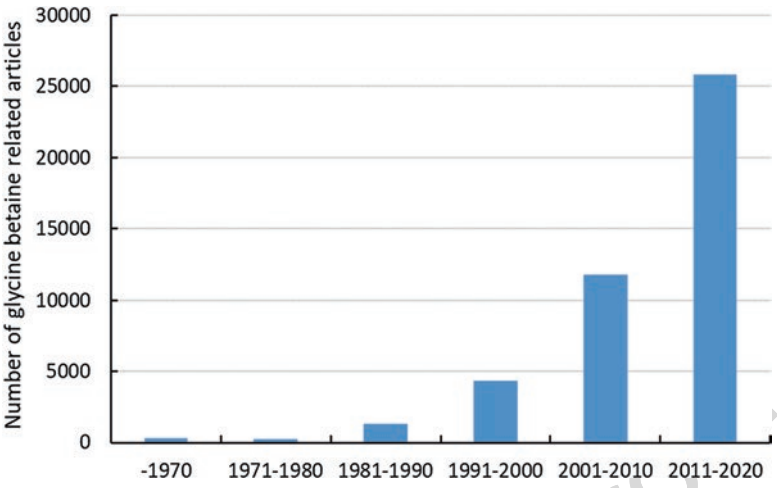

Fig. 1 The number of scientific articles containing the word "glycinebetaine" published in different decades based on a search in Google Scholar

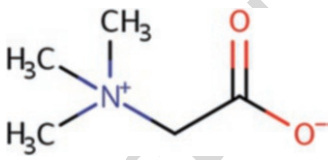

Fig. 2 Chemical structure of GB. GB has a zwitterionic nature as it possesses both negative (−) and positive (+) charges

duction (Xu et al. 2018 and cited literature). Accordingly, under abiotic stresses, increased ion concentration (e.g., Ca^{2+} and Na^{+}) can be detected by mitogen-activated protein kinase (MAPK), phospholipase D, and some proteins bound to the plasma membrane. MAPK signaling pathways transduce the stress signals which subsequently activate BADH and ROS-scavenging enzymes, such as peroxidase, catalase, superoxide dismutase, ascorbate peroxidase, and lipoxygenase. Finally, BADH accelerates the oxidation of betaine aldehyde to glycinebetaine. Within 24 h, GB is translocated via the phloem throughout the plant, especially to the youngest and developing plant parts (Mäkelä et al. 1996).

BADH gene expression can also be regulated by abscisic acid (ABA) (Kurepin et al. 2015 and cited literature). Kurepin et al. (2015) suggested that the close interaction and synergistic physiological effects of GB and ABA, resulting in increased freezing tolerance and a dwarf phenotype, are the major factors leading to effective cold acclimation of higher plants. However, Xu et al. (2018) concluded that the expression of BADH may also be ABA-independent. Instead, they proposed that jasmonate biosynthesis plays a dominant role in the activation of BADH and CMO under osmotic stress.

3.1 Endogenous Glycinebetaine and Osmotic Stress

Soil salinity is among the main abiotic stresses restricting crop production, and thus major efforts have been made to improve the salinity tolerance of crops. At first, the effect of soil salinity on plants is comparable to water deficit due to low water potential, and the effects of ion-specific toxicity only appear later, in the second phase (Munns 2010). Accumulation of osmolytes, such as GB, allows additional water uptake and therefore buffers the immediate effects of water deficit.

While some crops, especially Amaranthaceae and Poaceae, accumulate GB, in the majority of cases the accumulated concentrations for the whole plant might not be physiologically significant. Red beet (*Beta vulgaris* L.) is salt tolerant and one of the crops which accumulate GB as a response to increasing cell Na^+ concentration, among other triggers (Subbarao et al. 2001). In red beet subjected to salt stress, the leaf water content did not vary markedly even though the Na concentration increased up to 400 mol m^{-3} in the leaves and leaf osmotic potential increased. This was due to a simultaneous increase in GB concentration, contributing 50–60% to the leaf osmotic potential in the cytoplasm. Increasing GB concentration also correlates with maintenance of photosynthesis and chlorophyll fluorescence (Subbarao et al. 2001). According to Leigh et al. (1981), in red beet 26–84% of GB is localized in the cytoplasm, and the concentration in the cytoplasm varies between 46 and 467 mol m^{-3}, whereas the concentration in the vacuole ranges between 2.7 and 17.8 mol m^{-3}. Furthermore, Robinson and Jones (1986) showed that in salt-stressed spinach (*Spinacia oleracea* L.), at least 40% of GB is localized in chloroplasts, contributing 36% of the leaf osmotic potential. Thus, when GB concentration is calculated according to cytoplasm volume, its physiological role becomes significant.

Grumet and Hanson (1986) stated that GB has a marked role in osmoregulation of barley (*Hordeum vulgare* L.) by maintaining osmotic potential. Later, it was found that GB is the main compatible solute accumulating specifically in young barley leaves (Hattori et al. 2009). GB synthesis is localized in the vascular tissues of leaves and in the pericycle of roots. This is based on the finding that signal transcripts of *BBD2* gene increased in the vascular parenchyma cells of leaves and in the root pericycle. *BBD2*, more abundant in barley, has a 2000-fold affinity for betaine aldehyde in comparison to *BBD1*.

In durum wheat (*Triticum durum* Desf.), GB is one of the major osmolytes accumulating under prolonged salinity, accumulating especially in young leaves (Carillo et al. 2008). Interestingly, GB accumulation has been shown to correlate positively with glutamate synthase activity in young leaves, though it was independent of nitrogen nutrition of the plant. According to Khan et al. (2012), GB accumulation in salt-stressed bread wheat (*Triticum aestivum* L.) is linked to both increased salt tolerance and ethylene evolution. These changes are related to the maintenance of photosynthesis fluorescence and lower hydrogen peroxide content.

Accumulation of GB can also be cultivar or genotype specific. In cereals, the species and cultivar differences in GB accumulation are marked. For example, some

genotypes of sorghum (*Sorghum bicolor* (L.) Moench) and maize (*Zea mays* L.) accumulate GB, whereas others do not (Grote et al. 1994; Saneoka et al. 1995). However, even cereal cultivars that do not accumulate detectable concentrations of GB have active BADH and BADH protein in leaves (Ishitani et al. 1993). Peel et al. (2010) compared the GB metabolism in GB-accumulating and non-accumulating maize and sorghum. They concluded that GB deficiency in non-accumulating cereals could result either due to limited availability of choline or lack of choline transporter. The presence of genotypic differences in GB accumulation may explain at least partly the occurrence of stress-tolerant and stress-susceptible genotypes within individual plant species.

Some legumes, including mung bean (*Vigna radiata* (L.) R. Wilczek), also accumulate GB as a response to abiotic stresses. Misra and Gupta (2005) showed a salt-tolerant mung bean cultivar accumulating a higher concentration of GB under salt treatment in comparison to a salt-sensitive cultivar. Similarly, chlorophyll remained higher in the salt-tolerant cultivar. Khan et al. (2014) found that under salinity, GB accumulation in mung beans was induced by salicylic acid, which increased methionine production and suppressed ethylene production, opposite to the results of their barley study (Khan et al. 2012). When salicylic acid inhibits ethylene production, the metabolite of methionine and precursor of ethylene, s-adenosyl methionine, donates a methyl group to GB synthesis and promotes GB synthesis.

3.2 *Endogenous Glycinebetaine and Temperature Stress*

Yang et al. (1996) tested the high temperature (45 °C) tolerance of near-isogenic maize lines which differ in their ability to accumulate GB. The leaves of GB accumulators had less membrane damage, and the temperature threshold difference between the lines was 2 °C. Furthermore, the GB accumulators showed better thermostability of the PSII electron chain. These results indicate that GB might play a role in the protection of plasma membranes.

At the other extreme, Kishitani et al. (1994) studied the role of GB on the freezing tolerance of barley leaves by using near-isogenic lines whose ability to accumulate GB ranges from 10 to 90 μmol g^{-1} DM. After acclimation at 5 °C and freezing at −5 °C, the youngest leaves with the highest GB concentration survived, whereas the oldest leaves with the lowest concentration of GB died. Thus, it was concluded that GB plays a marked role in cold acclimation against freezing injury in young barley leaves.

Cooling is a useful storage method commonly employed to prolong postharvest life of plant produce. It reduces postharvest decay of tissues during transportation to distant markets and assures the availability of good quality produce to consumers for an extended period. However, many fruits and vegetables are chilling sensitive and highly vulnerable to chilling injury during cold storage at low temperatures, e.g., below 8 °C. The severe development of chilling injury decreases produce quality, for example, in appearance, texture, flavor, and nutrition. Unfavorable chilling

temperature directly promotes membrane phase transition from fluid liquid crystalline to rigid solid gel, leading to a decline in the membrane selective permeability. In addition, chilling temperature as an oxidative stress factor indirectly promotes ROS accumulation, resulting in the peroxidation of unsaturated fatty acids in plant membranes. Recent reports, summarized here, indicate that GB is a useful molecule for reducing chilling injuries in several fruits. The mechanisms seem to be similar to those found in whole-plant studies and in their response to common stresses.

Jin et al. (2015) studied the influence of low-temperature conditioning treatment (at 10 °C for 6 days) on chilling injury, GB concentration, and energy metabolism in loquat fruit (*Eriobotrya japonica* (Thunb.) Lindl) stored at 1 °C. Their results indicate that low-temperature conditioning treatment significantly reduces chilling injury, ion leakage, and malondialdehyde content in loquat fruit. BADH activity and endogenous GB content in loquats treated with low-temperature conditioning were significantly higher than in control fruit. Moreover, low-temperature conditioning treatment induced activities of energy metabolism-associated enzymes, including H^+-adenosine triphosphatase, Ca^{2+}-adenosine triphosphatase, succinic dehydrogenase, and cytochrome c oxidase. The low-temperature conditioning treatment clearly triggered higher levels of ATP content and energy charge, and together these results show that low-temperature conditioning may alleviate chilling injury and improve chilling tolerance of loquat fruit by enhancing endogenous GB accumulation and energy status.

Yao et al. (2018) suggested that GB can ameliorate the chilling injury in zucchini (*Cucurbita pepo* L.) fruit. The effects of GB treatment were associated with an accumulation of proline and a reduction in lipid peroxidation. In addition, GB-treated fruit also showed lower levels of palmitic acid and stearic acid, and lower lipoxygenase and plant phospholipase D activities, but higher activity levels of enzymes related to proline metabolism. The gene expression and antioxidant enzyme activities of superoxide dismutase, catalase, and ascorbate peroxidase in GB-treated fruit were significantly higher than that of control fruit. Thus, GB could alleviate chilling injury in cold-stored zucchini fruit through improved antioxidant enzymatic mechanisms in addition to the involvement of fatty acid metabolism.

Recently, Razavi et al. (2018) reported that in hawthorn (*Crataegus monogyna* Jacq.) fruits, GB applied by immersion for 15 min at 20 °C resulted in a steady increase of endogenous GB accumulation during storage at 1 °C for 20 days. This accumulation was then associated with delayed fruit pitting development. They also found that higher endogenous GB accumulation correlated with higher activity of antioxidant enzymes, such as superoxide dismutase, catalase, and ascorbate peroxidase, leading to lower buildup of hydrogen peroxide. In addition, fruits treated with GB exhibited significantly higher content of phenols, flavonoids, and anthocyanins, which was due to the higher activity of phenylalanine ammonia lyase enzyme. Furthermore, the observed higher ascorbic acid accumulation in GB-treated fruits resulted in higher 1,1-diphenyl-2-picrylhydrazyl-scavenging capacity during storage at 1 °C for 20 days. The authors propose that GB treatment is a useful strategy for attenuating chilling injury of hawthorn fruit due to lower ROS accumulation. Moreover, the application of GB could be favorable in terms of maintaining nutri-

tional quality of hawthorn fruit because it increases the level of antioxidant molecules, beneficial for human health. Wang et al. (2019) also showed that GB could enhance the chilling tolerance of peach (*Prunus persica* (L.) Batsch) fruits through the regulation of phenolic and sugar metabolism, leading to the maintenance of high levels of individual phenolic and sucrose content.

4 Glycinebetaine and Transgenesis Approaches to Improve Plant Stress Tolerance

Plants cope with abiotic stresses by activating response pathways that result in redirection of resources from growth toward resistance. Abiotic stress tolerance is often manifested in the accumulation of protective enzymes and metabolites. Primary metabolites are conserved molecules required for normal growth and development, while secondary metabolites are related more to signaling and are more diverse among different species. Understanding metabolic fluxes in plant cells in response to many environmental factors requires genome-wide systems approaches. Plant metabolomics addresses the biochemistry and molecular mechanisms of plant responses to cope with osmotic stress. It combines sample separation by liquid or gas chromatography and the detection of metabolites based on their ion mass and charge. In general, metabolomic analysis is less dependent on genomic information than many other molecular omics studies, such as transcriptomics or proteomics. Therefore, this technology is accessible for a wide range of species.

With regard to the accumulation of osmolytes, such as GB, plant species are recognized as GB accumulators or non-accumulators. Transgenesis has introduced the GB pathway into many non-accumulator species and increased GB levels in GB-accumulating species. In this chapter, we summarize the current understanding of the challenges in genetically engineering GB accumulation in plants.

4.1 Transgenesis for Improved GB Levels

In plants, biosynthesis of GB is a simple two-step reaction cascade involving choline oxidation reaction by CMO followed by oxidation of the resulting BADH. In *Escherichia coli*, the BetA and BetB enzymes mediate these two reactions. The COD (*Arthrobacter globiformis*) and COX (*Arthrobacter pascens*) pathways represent prokaryotic choline oxidases that mediate direct conversion of choline to GB (Sakamoto and Murata 2001). Despite these straightforward reaction cascades, transgenesis approaches have proven challenging to optimize for obtaining physiologically relevant GB osmolyte levels. Transgenesis approaches in plant species lacking a functional GB biosynthesis pathway have utilized both prokaryotic and eukaryotic genes. Utilizing genes from a prokaryotic origin reduces considerations of translational and posttranslational modifications. Standard overexpression of one

of the biosynthetic enzymes aims to increase levels of gene expression in the cell. Overexpression vectors usually harbor a 35S promoter and terminators together with antibiotic selection. Physiologically relevant levels for GB to act as an osmotic regulator range between tens of μM to hundreds of μM (Annunziata et al. 2019). GB accumulation at the level of 5 μmol g^{-1} DM, or down to 1 μmol g^{-1} FW, has also been suggested as promoting stress resistance as summarized in Khan et al. (2009) and Chen and Murata (2011). As stated earlier, this activity depends on the compartmentation of GB in cells.

In tobacco, overexpression of *E. coli BetA* (*CDH*) alone or together with *BetB* (*BADH*) conferred the transgenic plants with increased resistance to salt stress compared to wild-type plants (Holmström et al. 2000). Overexpression resulted in functional enzymes and enhanced the plant's ability to process betaine aldehyde, the toxic intermediate of the GB synthesis pathway. The GB levels, however, remained at a low level (40–80 nmol g^{-1} FW), suggesting that the stress-protective effect was not due to osmoregulation. Mild accumulation of GB might still be adequate to protect protein complexes and membranes, for example, in chloroplasts.

Cotton cv. Luyuan890 has been engineered to constitutively overexpress the *betA* gene from *E. coli* (Lv et al. 2007). In wild-type plants, the GB levels were already physiologically relevant, with high levels of approximately 100 μmol g^{-1} DM. The *betA* transgenic lines accumulated GB at over 130 μmol g^{-1} DM, and their drought resistance and physiological performance were analyzed. Four out of five of the lines were shown to perform better for maintenance of osmotic potential and relative water content.

In overexpression approaches, *codA* from *Arthrobacter globiformis* has been most popular, although the resulting GB levels usually remain moderate (Khan et al. 2009; Chen and Murata 2011). In tomato (*Solanum lycopersicum* L.) transgenesis, *codA* from *Arthrobacter globiformis* was used to mediate direct choline conversion to GB, in contrast to two-step biosynthesis (Wei et al. 2017; Khan et al. 2009). Overexpression in tomato cv. Moneymaker resulted in L1, L2, and L3 lines with minor increases in GB accumulation of up to 2 μmol g^{-1} DM. Following NaCl treatment, GB accumulation reached 5–6 μmol g^{-1} DM and was shown during stress to increase photosynthetic rate and antioxidant enzyme activity and to reduce ROS accumulation (Wei et al. 2017). Changes in Na^+/K^+ ion balances were observed in the transgenic lines, resulting from increased Na^+ exclusion and decreased K^+ efflux. These effects were mediated through ion channel gene expression. It is proposed that GB could promote salt tolerance through regulation of the respective channels and transporters. In addition, GB may enhance antioxidant enzyme activities and thereby alleviate ROS responses and damage to photosynthesis in the leaves. Salt stress is known to impair photosynthesis, and it has been suggested that the positive impact of GB on photosynthesis results from better osmotic adjustment and prevention of stomatal closure (Lv et al. 2007).

A second study on the tomato cv. Moneymaker *codA* transgenic lines (*codA Arthrobacter globiformis*) with relatively low GB accumulation (up to 2.5 μmol g^{-1} FW) addressed the role of GB in abiotic stress resulting from phosphate starvation (Li et al. 2019). The transgenics were able to maintain P_i/H^+ co-transport, and the

gene expression of the PHO regulon was also modified, and photosynthetic rates remained high. In the transgenic lines, growth was enhanced as indicated by increased fresh weight and shoot and root size, while stress responses such as anthocyanin accumulation were lower compared to wild type. Here, moderate GB accumulation mediated physiological and biochemical changes so that environmental adaptation processes were impacted. GB biosynthesis by COD/COX results in side product hydrogen peroxide accumulation, which operates in redox sensing, signaling, and regulation in eukaryotic cells (Sies 2017). In Arabidopsis (*Arabidopsis thaliana* L.) transformed with the *codA* gene for choline oxidase, accumulation of steady-state hydrogen peroxide was detected at the level of 960 nmol g^{-1} FW compared to 750 nmol g^{-1} FW in wild type (Hayashi et al. 1997; Sakamoto and Murata 2001). Part of the observed effects from COD/COX transgenesis thus might be due to such alternative responses.

Transgenic wheat line (T6) has been generated to overexpress the *Atriplex hortensis* L. *BADH* gene in the shi4185 line. In the wild-type wheat line, GB concentration is already at a high level of 75 μmol g^{-1}; *BADH* overexpression caused this to increase to 100 μmol g^{-1} DM (Wang et al. 2010). A similar increase was seen in the wild type after drought treatment. In the study, drought, heat, and their combination were tested in the wild-type and overexpressing line. The responses in the T6 line appeared milder compared to wild type for most of the parameters measured for the three replicates. The heat stress effects on transpiration and stomatal conductance deviated from drought and combination responses.

Interestingly, most transgenic plants can utilize exogenously applied choline, and GB levels remain stress-inducible in transgenic lines even if transgenes are driven by a constitutive 35S promoter (Lv et al. 2007). This suggests that GB biosynthesis is further promoted by the stress condition. This regulation can be at the transcript level or at the post-translational level. Conversely, this also suggests that transgenesis approaches have not addressed all the components involved. In transgenesis of non-accumulators that lack all functional GB biosynthesis enzymes, overexpression of only one component often leaves the GB accumulation levels moderate. Unbalanced expression of biosynthetic enzymes from the GB pathway can create different cellular and metabolic imbalances (Hare et al. 1998; Gage et al. 2003; Chen and Murata 2011). For example, BADH is not a substrate-specific enzyme and has been associated with diverse aldehydes (Trossat et al. 1997; Muñoz-Clares et al. 2014). The alternative reaction cascades of the GB biosynthesis enzymes can result in competition between substrates and cause side effects, for example, in polyamine metabolism, possibly resulting in new phenotypes (Trossat et al. 1997).

Taken together, transgenesis of only one gene from a biosynthetic pathway is usually not enough to achieve the intended outcome. Limiting factors for GB biosynthesis can be the availability of choline, activity of the biosynthetic enzymes and their specificities toward the substrates, as well as the subcellular localization of the enzymes and their respective substrates (Huang et al. 2000; Nuccio et al. 1998, 2000; Kumar et al. 2004; Muñoz-Clares et al. 2014; Carrillo-Campos et al. 2018). Modifications to the single-gene overexpression approaches are represented by

gene stacking, a transgenesis method in which combinations of constructs harbor more than one gene and can be transferred under one selection (Zorrilla-López et al. 2013). In principle, gene stacking would allow transferring all the limiting factors from a biosynthesis pathway in one or consecutive events. Hence, gene stacking could solve some of the bottlenecks in transgenesis for GB accumulation.

4.2 Considerations for CMO and BADH Isoenzymes

Significant sequence-specific differences have been discovered in the GB biosynthesis isoenzymes. Phylogenetic studies show that all land plant species have genes encoding for CMO enzymes (Carrillo-Campos et al. 2018). The CMO genes are present in two clades, CMO1 and CMO2, whereby CMO2 has diverged from the CMO1 after genome duplication. CMO2-type enzymes have evolved at a fast rate and are present in GB-accumulating plant species, such as spinach (Fig. 3). Homology modeling and docking simulations have shown that the CMO2 active site has three aromatic residues and a glutamate that allow efficient interaction with the substrate, choline. The four critical amino acids of CMO2 that confer substrate specificity for choline are indicated in Fig. 3. Such binding capacity toward choline is lacking from the CMO1-type isoenzymes, the isozymes that prevail in GB non-accumulators. Spinach also has CMO1-type enzymes that don't utilize choline but act as oxygenases on different substrates. It would be interesting to verify which spinach CMO form was used in the transgenesis approaches that resulted in low GB accumulation (Shirasawa et al. 2006).

Functional isoenzyme differences have also been discovered for the second step of GB biosynthesis, in the BADH isozymes (Muñoz-Clares et al. 2014). BADH isoenzymes belong to the family 10 of aldehyde dehydrogenases, but only certain ALDH10 enzymes appear to have BADH activity on BAL. Phylogenetic analysis has shown that in spinach, a GB accumulator, the BADH enzyme has a particular amino acid at position 441 (alanine A441), while GB non-accumulators, such as *Arabidopsis*, have isoleucine at this position (Fig. 4). The amino acid in position 441 (painted gray in Fig. 3) appears to determine if enzymes are able to oxidize BAL into GB. These structure functional discoveries in GB biosynthesis enzymes are likely to influence the success of future transgenesis approaches for enhancing GB production in plants.

4.3 Chloroplast Targeted Transgenesis for Optimized GB Production

Endogenous GB biosynthesis is compartmentalized within the chloroplast. Targeting GB accumulation directly in the chloroplast can facilitate correct enzyme conformation in the correct subcellular compartment. Chloroplast genetic engineering has

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Spinacia_CMO2          MMAASASATTMLLKYPTTVCG-IPNPSSNNNNDPSNNIASIPQNTTNPTLKSRTPNKITT   59
Spinacia_CMO1          MS------------ITHSIT---QNPLTNHVTLQSFGNNFIPK-------IERFPNRIHQ   38
Arabidopsis_CMO1       MMTT----------LTATVPEFLPPSLKSTRGYFNSHSEFGVS-------ISKFSRRRFH   43
                       *               ::        ..     .       .        .:  .:

Spinacia_CMO2          NAVAAPSFPSLTTTTPSSIQSLVHEFDPQIPPEDAHTPPSSWYTEPAFYSHELERIFYKG   119
Spinacia_CMO1          APIKLTKC-LSNSSSIQSTHKIAHEFDPNIPIEEAQTPPCSWYSDPEFYSHEIDRVFYSG   97
Arabidopsis_CMO1       NPTR--------VFAVSDISKLVTEFDPKIPLERASTPPSSWYTDPQFYSFELDRVFYGG   95
                                  : ..  .:. ****:** * * ***.***::* ***.*::*:** *

Spinacia_CMO2          WQVAGISDQIKEPNQYFTGSLGNVEYLVSRDGEGKVHAFHNVCTHRASILACGSGKKSCF   179
Spinacia_CMO1          WRVVGCVDQIKNAHDYFTGRLGNVEYVICRDGVGKIHAFHNVCRHHASILAYGSGRKTCF   157
Arabidopsis_CMO1       WQAVGYSDQIKESRDFFTGRLGDVDFVVCRDENGKIHAFHNVCSHHASILASGNGRKSCF   155
                       *:..*  ****: .::*** **:*::::.**  **:******* *:***** *.*:*:**

Spinacia_CMO2          VCPYHGWVYGMDGSLAKASKAKPEQNLDPKELGLVPLKVAVWGPFVLISLDRSLEEG---   236
Spinacia_CMO1          VCPYHGWTYGLEGNLLKAPRITGLRNFNPKEYGLVPINVATWGPFVVVNLSSSEEE---V   214
Arabidopsis_CMO1       VCLYHGWTYSLSGSLVKATRMSGIQNFSLSEMGLKPLRVAVWGPFVLLKVTAATSRKGEV   215
                       ** ****.*.:.*.* ** : .  :*:. .* ** *:.**.*****::.:  : ..

Spinacia_CMO2          ---GDVGTEWLGTSAEDVKAHAFDPSLQFIHRSELPMESNWKIFSDNYLDSSYHVPYAHK   293
Spinacia_CMO1          -DYGNMENDWLGGSADLLSINGVDTSLSYICRREYTLECNWKVFCDNYLDGGYHVPYAHK   273
Arabidopsis_CMO1       ETDELVASEWLGTSVGRLSQGGVDSPLSYICRREYTIDCNWKVFCDNYLDGGYHVPYAHK   275
                            : .:*** *.  :.  ..*  *.:* * *  ::.***:*.*****..********

Spinacia_CMO2          YYATELNFDTYDTQMIENVTIQRVEGSS-NKPDGFDRVGIQAFYAFAYPNFAVERYGPWM   352
Spinacia_CMO1          NLASGLNLDSYSTEMFEKVSIQRCASSSTETGEDFDRLGSKALYAFVYPNFMINRYGPWM   333
Arabidopsis_CMO1       GLMSGLDLETYSTTIFEKVSIQECGGGSKVGEDGFDRLGSRALYAFVYPNFMINRYGPWM   335
                          : *::::*.* ::*:*:**.  ..*    :.***:* :*:***.**** ::******

Spinacia_CMO2          TTMHIHPLGPRKCKLVVDYYIENSMLDDKDYIEKGIAINDNVQREDVVLCESVQRGLETP   412
Spinacia_CMO1          DTNLVIPLGPRKCQVVFDYFLDASLKDDKAFIERSLKDSEEVQIEDIMLCEGVQRGLESP   393
Arabidopsis_CMO1       DTNLVLPLGPRKCKVVFDYFLDPSLKDDEAFIKRSLEESDRVQMEDVMLCESVQRGLESQ   395
                        *  : *******::*.**::: *: **: :*::.: .:.** **::***.******:

Spinacia_CMO2          AYRSGRYVMPIEKGIHHFHCWLQQTLK-----    439
Spinacia_CMO1          AYNTGRYAPTLEKPMHHFHCLLYRNLTEQTLQF   426
Arabidopsis_CMO1       AYDKGRYAL-VEKPMHHFHCLLHHNLKL-----   422
                       ** .***.  :** :***** .  :.*.
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Fig. 3 Spinacia CMO1 (XP_021866412.1) and CMO2 (ABN43460.1) amino acid sequence alignment with Arabidopsis CMO1 amino acid sequence by Clustal Omega tool (Madeira et al. 2019). Spinacia CMO2 functional motives for choline oxidation as indicated by Carrillo-Campos et al. (2018) are underlined and painted in grey. Stars under sequences indicate shared amino acids, single and double dot indicates semi- and conservative amino acids, respectively, no sign indicates non-conservative amino acid

many benefits over nuclear transgenesis (Kumar et al. 2004). Direct chloroplast genome transgenesis and efficient transgene expression have been achieved with appropriate regulatory sequences for both selection and the gene of interest. Homologous recombination in the chloroplast genome requires extensive flanking sequences around the gene of interest. The carrot (*Daucus carota* subsp. *sativus* (Hoffm.) Schübl. & G. Martens)-specific transformation vector, pDD-DC-aadA/badh, harbored aadA and badh sequences regulated by the 5'ribosome-binding site region of the bacteriophage T7 gene 10 leader to facilitate expression in green and non-green tissues. Similarly, the promoter sequence was designed to harbor binding sites for both plastid- and nuclear-encoded RNA polymerases. Transgenesis was performed by particle bombardment of a yellow carrot cell culture. The untransformed cell remained yellow in color while transformed cells turned green, allowing selection without a selectable marker. The method was completed with the successful regeneration of mature plants through somatic embryogenesis. Directing

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Spinacia_BADH     MAFPIPARQLFIDGEWREPIKKNRIPVINPSTEEIIGDIPAATAEDVEVAVVAARRAFRR        60
Arabidopsis_ALDH  MAIPMPTRQLFIDGEWREPILKKRIPIVNPATEEVIGDIPAATTEDVDVAVNAARRALSR        60
                  **:*:*:************** *:***::**:***:********:***:*** *****: *

Spinacia_BADH     N---NWSATSGAHRATYLRAIAAKITEKKDHFVKLETIDSGKPFDEAVLDIDDVASCFEY        117
Arabidopsis_ALDH  NKGKDWAKAPGAVRAKYLRAIAAKVNERKTDLAKLEALDCGKPLDEAVWDMDDVAGCFEF        120
                  *   :*: : ** **.********:.*:* .:.***::*.***:**** *:****.***:

Spinacia_BADH     FAGQAEALDGKQKAPVTLPMERFKSHVLRQPLGVVGLISPWNYPLLMATWKIAPALAAGC        177
Arabidopsis_ALDH  YADLAEGLDAKQKAPVSLPMESFKSYVLKQPLGVVGLITPWNYPLLMAVWKVAPSLAAGC        180
                  :*. **.**.******:**** ***:**:*********:*********.**:**:*****

Spinacia_BADH     TAVLKPSELASVTCLEFGEVCNEVGLPPGVLNILTGLGPDAGAPLVSHPDVDKIAFTGSS        237
Arabidopsis_ALDH  TAILKPSELASVTCLELADICREVGLPPGVLNVLTGFGSEAGAPLASHPGVDKIAFTGSF        240
                  **:**************:.:::*.**********:***:* :*****.***.*********

Spinacia_BADH     ATGSKVMASAAQLVKPVTLELGGKSPIVVFEDVDIDKVVEWTIFGCFWTNGQIXCSATSR        297
Arabidopsis_ALDH  ATGSKVMTAAAQLVKPVSMELGGKSPLIVFDDVDLDKAAEWALFGCFWTNGQI-CSATSR        299
                  *******::********::*******::**:***:**..**::********** ******

Spinacia_BADH     LLVHESIAAEFVDKLVKWTKNIKISDPFEEGCRLGPVISKGQYDKIMKFISTAKSEGATI        357
Arabidopsis_ALDH  LLVHESIASEFIEKLVKWSKNIKISDPMEEGCRLGPVVSKGQYEKILKFISTAKSEGATI        359
                  ********:**::******:*********:*********:******:**:**************

Spinacia_BADH     LYGGSRPEHLKKGYYIEPTIVTDISTSMQIWKEEVFGPVLCVKTFSSEDEAIALANDTEY        417
Arabidopsis_ALDH  LHGGSRPEHLEKGFFIEPTIITDVTTSMQIWREEVFGPVLCVKTFASEDEAIELANDSHY        419
                  *:*********:**::******:**::******:*************:****** ****:.*

Spinacia_BADH     GLAAAVFSNDLERCERITKALEVGAVWVNCSQPCFVQAPWGGIKRSGFGRELGEWGIQNY        477
Arabidopsis_ALDH  GLGAAVISNDTERCDRISEAFEAGIVWINCSQPCFTQAPWGGVKRSGFGRELGEWGLDNY        479
                  **.***:*** ***:**::*:*.* **:********.******:*************::**

Spinacia_BADH     LNIKQVTQDISDEPWGWYKSP-    498
Arabidopsis_ALDH  LSVKQVTLYTSNDPWGWYKSPN    501
                  *.:****   *::*********
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Fig. 4 Amino acid sequence alignment of Spinacia BADH protein (ACM67311.1) with Arabidopsis non-BAL form ALDH (10A8) enzyme. The critical amino acid (441A or 441C) for BADH BAL activity as shown by Muñoz-Clares et al. (2014) is underlined and painted in grey. Stars under sequences indicate shared amino acids, single and double dot indicates semi- and conservative amino acids, respectively, no sign indicates non-conservative amino acid

BADH gene overexpression in carrot chloroplasts resulted in the highest salt tolerance of up to 400 mM NaCl (Kumar et al. 2004).

While enzyme activities and substrate specificities are fundamental for biosynthetic pathways, subcellular localization of the biosynthesis enzymes also plays a significant role. In GB accumulators, all functional biosynthesis enzymes are present in the correct subcellular localization, and the substrate choline is also available. Biosynthetic enzymes of GB are encoded in the plant genome while GB biosynthesis takes place in chloroplasts. The substrate choline is transported into the chloroplasts via nuclear pores. Successful transgenesis for enhanced GB production would thus require enhanced levels of both the transgene products and the substrate choline (Nuccio et al. 2000; McNeil et al. 2000). Gene expression levels and organ-specific expression patterns of biosynthetic enzymes can be regulated by promoter elements, but localization of the gene products is also affected by signaling peptides, called transit signals. Overexpression of biosynthetic enzyme-encoding genes can be accompanied by signal sequences to translocate the gene products into chloroplasts. The study of Nuccio et al. (2000) represents a rigorous effort to optimize the expression, localization, and posttranslational modifications of GB biosynthesis enzymes.

In the study, a 100-fold higher CMO activity was achieved in tobacco chloroplasts, yet the levels of GB remained at a low level. The availability of the substrate, choline, was shown to be the limiting factor. Plants engineered to express CMO in chloroplasts failed to produce GB even at high gene expression levels, while transgenic lines expressing CMO in the cytoplasm accumulated significantly more GB. It was shown that poor choline transport into chloroplasts caused the lack of GB accumulation in the chloroplast-targeted CMO line. These studies are a reminder of the importance of assessing all the components along the pathway. To further promote choline availability for GB biosynthesis, choline biosynthesis could also be enhanced through transgenesis. Recently, a newly identified factor, GB1, was shown to promote GB accumulation at high levels in different maize cultivars (Castiglioni et al. 2018). Overexpression of this fatty acid hydroxylase superfamily protein was speculated to be involved in choline biosynthesis and/or transported into chloroplasts. Future work will confirm GB1 function, but the availability of choline clearly represents a critical limiting factor for GB accumulation.

5 Conclusions and Future Perspectives

The amount of scientific literature related to GB is accumulating quickly, yet our knowledge of the mechanisms by which GB affects crop stress tolerance remain partly unknown. It is proposed that GB acts as a compatible solute in plants with two major roles. The first role of GB involves the regulation of osmotic balance via acting as a conventional osmolyte. The second one includes the maintenance of normal cell metabolism under stress conditions and thus acting on ROS scavenging, macromolecule protection, and carbon and N reserves.

Some of the proposed effects of GB might be the result of alternative metabolic routes caused by imbalanced metabolic engineering. Integrated omics analysis combining transcriptomic, proteomic, and metabolomic studies on the transgenic lines could shed light on the complete picture of the GB accumulation profiles of the different transgenesis approaches.

There are many limiting factors that seemingly influence GB accumulation in transgenic plants. Gene stacking as a transgenesis strategy could solve some of the bottlenecks in improving GB accumulation. The significant structure function discoveries in the GB biosynthesis isoenzymes are especially likely to drive the success of future GB transgenesis approaches in plants. It could also be considered whether marker-assisted selection could prove useful in the isoenzyme approach.

In future, more attention should be paid to investigating the mechanisms by which GB affects plant growth and metabolism instead of simply testing new plant species.

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