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Review

The Role of the γ-Aminobutyric Acid (GABA) in Plant Salt Stress Tolerance

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Abstract: γ-Aminobutyric acid (GABA) is a non-protein amino acid that accumulates in many plant species in response to environmental stress. A number of reverse-genetic experiments and omics analyses have revealed positive relationships between GABA levels and tolerance to stresses. Furthermore, the application of exogenous GABA has been demonstrated to effectively reduce ROS levels, enhance membrane stability and modulate phytohormones cross-talk, thus improving tolerance against multiple stresses. However, molecular mechanisms regulating GABA homeostasis and physiological functions in plants remain largely unclear. In this review, we focus on the recent achievements in deciphering the role of genetic manipulations to modulate endogenous GABA levels and the exogenous application of GABA and associated metabolites to improve tolerance to salt stress. Finally, we discuss the role of GABA in the regulation of ion homeostasis in high-salinity conditions. These findings have laid the groundwork for future studies to explore the genetic, physiological, and molecular mechanisms of GABA-mediated improvements in plant productivity under high-salt environmental conditions.

Keywords: γ-aminobutyric acid; GABA; salt stress; salt tolerance; ROS levels; membrane stability; phytohormones cross-talk ion homeostasis; improvement of plant productivity

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1. Introduction

1.1. Physiological Effects of Salt Stress on Plants

Salt stress affects over 20% of the world's cultivated land and 60% of irrigated land, which leads to both reduced crop productivity and soil degradation [1]. Taking into account the increasing human population of the world, global food production has to rise significantly through improved tolerance to salinity stress. Unfortunately, the current progress is rather limited, even with the application of novel genetic engineering methods and genome-wide studies [2,3]. Despite decades of intensive research, many of the molecular mechanisms involved in salt tolerance are still unknown. The complexity of participating metabolic processes controlled by the interplay of the vast array of genes does not allow a desirable balance to be achieved between adequate salinity tolerance and energy supplies for growth-related and defense mechanisms [4].

Salt stress results in diverse metabolic and physiological changes, which eventually reduce crop productivity. The primary effect of soil salinity is mediated through osmotic stress and ion toxicity, leading to oxidative stress and nutritional imbalance. In the next stage, hyperosmotic stress disrupts normal water uptake by the root and increases leaf water loss [5]. Hyperionic stress, the second major aspect of salt stress, causes dysfunction of cellular ion homeostasis via direct inhibition of various cellular enzymes [6]. Photosynthesis is the major process to procure energy and fix it in carbon compounds for subsequent use for plant growth and maintenance. Salt stress greatly reduces the amount of acquired energy due to the decreased rate of photosynthesis and because of the reallocation of

some energy to tolerance and defense mechanisms. Additionally, high salinity damages chloroplast proteins, lowers stomatal conductance, alters respiration rates, and increases the accumulation of damaging ROS [7]. Subsequently, ROS leads to increased lipid peroxidation on chloroplast and mitochondrial membranes and the production of toxic aldehydes (such as acrolein, 4-hydroxy-trans-2-nonenal and 4-hydroxy-trans-2-hexenal) [8]. Therefore, along with the decreased energy production, salinity enhances energy consumption and increases toxin output. On the other hand, the adaptation to and survival in low and moderate salt exposures require the coordination of many metabolic processes such as METC (mitochondrial electron transfer chain), TCA (tricarboxylic acid cycle), OPP (oxidative pentose phosphate) pathway, glycolysis, and others [4]. Thus, mitochondria and chloroplasts are proposed as the major organelles essential for salinity tolerance due to their involvement in ATP generation, ion exclusion and homeostasis, signal transduction, ROS detoxification, and the regulation of carbon balance [9].

1.2. The Role of GABA in Stress Responses

GABA (γ-aminobutyric acid) is a non-proteinogenic amino acid that is widely present in many plant tissues and organs as a metabolite and signaling molecule responsive to various biotic and abiotic stresses [10]. In the mammalian brain, GABA function as a major inhibitory neurotransmitter, whose activation results in membrane hyper-polarization in the central nervous systems and leads to the dampening of neuronal firing [11]. The roles of GABA in the brain and associated diseases have been extensively reviewed and will be omitted from this manuscript [12,13].

The involvement of GABA in stress responses has greatly stimulated interest in the application of exogenous compounds, genetic engineering, and breeding approaches to modulate GABA levels and improve tolerance to stresses [14]. Treatment with exogenous GABA increases the level of endogenous GABA and promotes a wide range of physiological and molecular responses: activating nitrogen assimilation, increasing GABA shunt to sustain mitochondrial tricarboxylic acid cycle and energy production, modulating the polyamines pathway, and increasing levels of antioxidants (CAT (catalase), ascorbic acid, SOD (superoxide dismutase), glutathione and others), and osmolytes (proline, sugars) [15]. Additionally, under certain conditions, GABA can directly bind different transporters and channels (such as ALMT (aluminum-activated malate transporter) and GORK (guard cell outward rectifying K^+), thus modulating ion homeostasis and improving stress tolerance [16]. We must note that the role of GABA in the plant growth, development, and biochemical and signaling aspects of GABA functioning are well documented in several excellent recent papers [9,17,18], and to avoid redundancy will not be covered in this review. In this review, we focus on the recent discoveries of the molecular mechanisms connecting GABA metabolism and plant salt stress tolerance. Additionally, different ways to modulate GABA levels (endogenous, with genetic manipulations, and exogenous, with treatments) to facilitate plant survival and productivity under salt stress conditions are discussed.

2. GABA Homeostasis in Plants

Since the original discovery of GABA in plants [19] and animals [20], several minor metabolic pathways have been discovered. However, the GABA shunt is considered the major pathway responsible for the synthesis and maintenance of optimal GABA levels (reviewed in [9]) (Figure 1, Figure S1 (simplified version)). There are three main enzymes in GABA biosynthesis: GAD (glutamate decarboxylase), GABA-T (GABA transaminase), and SSADH (succinic semi-aldehyde dehydrogenase). The α-decarboxylation of glutamate to GABA by GAD is an irreversible and rate-limiting step in GABA biosynthesis. Further, GABA-T converts GABA to succinic semi-aldehyde, and SSADH converts SSA to succinate (Figure 1). Through the different metabolites, GABA metabolism is connected to organelles and other metabolic pathways: glutamate and arginine (plastid), aspartate (vacuole), polyamine (peroxisome), and most importantly, via several metabolites, the mitochondrial

TCA cycle. GABA biosynthesis pathways from putrescine and other polyamines have also been reported and are considered alternative pathways (Figure 1) [21,22].

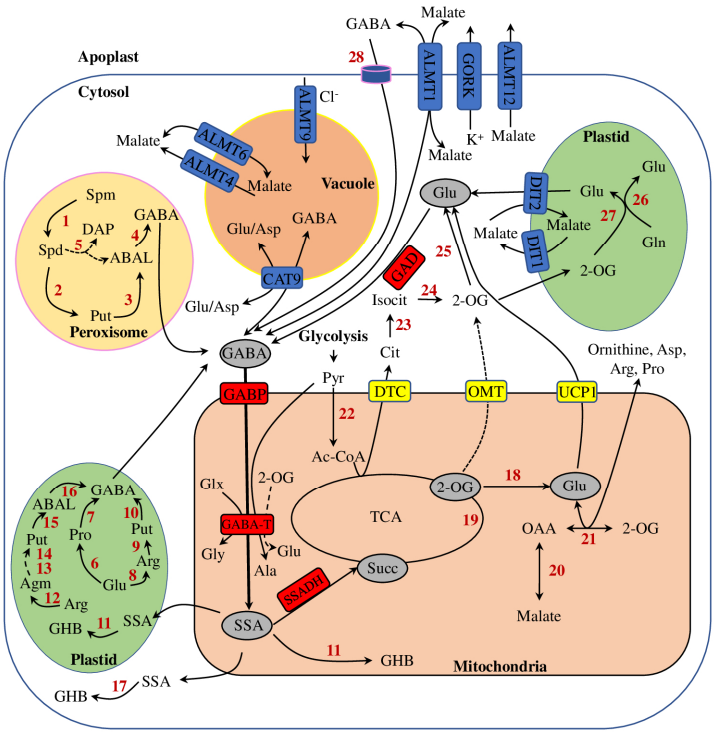

Figure 1. Representative model of *Arabidopsis thaliana* GABA metabolism and signaling. The grey ovals and red rectangles represent important metabolites and enzymes (GABA-T (GABA transaminase), GABP (GABA permease), SSADH (succinic semialdehyde dehydrogenase), and GAD (glutamate decarboxylase), respectively, in the GABA shunt. Dashed lines represent metabolic pathways with insufficient experimental data. The yellow squares represent transporters linking the TCA cycle back to the GABA shunt, and the small blue squares represent transporters linking the GABA shunt to other metabolic pathways and organelles (DIT1, DIT2, and CAT9). The big blue squares represent GABA-regulated transporters (ALMTs and GORK). Abbreviations: GABA, $\gamma-$aminobutyrate. **Peroxisome**. Spm—spermine; Spd—spermidine; Put—putrescine; ABAL—4-aminobutanal; DAP—1,3-diaminopropane; 1—PAO (polyamine oxidase 2–4); 2—PAO (polyamine oxidase 2,3); 3—AO1 (copper amine oxidase 1); 4—ALDH (NAD^+-dependent aldehyde dehydrogenase 10A8 and 10A9); 5—PAO (polyamine oxidase 2–4). **Plastid**. DIT—dicarboxylate translocator 1 and 2; Gln—glutamine; Glu—glutamate; Pro—proline; Arg—arginine; Agm—agmatine; SSA—succinic semialdehyde; GHB—$\gamma-$hydroxybutyrate; 6—Δ^{1-}pyrroline-5$-$carboxylate synthetase and Δ^{1-}pyrolline-5$-$carboxylate reductase; 7—spontaneous decarboxylation of proline to pyrrolidin$^{-1}-$yl; 8—urea cycle; 9—arginine decarboxylase, agmatine iminohydrolase and N$-$carbamoylputrescine amidohydrolase; 10—copper AO and ALDH10A8; 11—SSR2 (NADPH-dependent glyoxylate/succinic semialdehyde reductase); 12—arginine decarboxylase 1,2; 13—agmatine iminohydrolase; 14—N-carbamoylputrescine amidohydrolase; 15—copper AO; 16—ALDH10A8; 26—glutamine synthetase; 27—ferredoxin$-$dependent glutamate synthase. **Mitochondria**. Glx—Glyoxylate; Gly—glycine; GABA$-$T, GABA transaminase; Succ—succinate; Ac$-$CoA—acetyl$-$CoA; Ala—alanine; 2$-$OG—2$-$oxoglutarate; OAA—oxaloacetate; DTC—dicarboxylate/tricarboxylate carrier; OMT—2$-$oxoglutarate/malate translocator; UCP1—uncoupling protein 1; 18—GDH (glutamate dehydrogenase); 19—2$-$oxoglutarate dehydrogenase and succinyl$-$CoA ligase; 20—malate dehydrogenase; 21—aspartate transaminase; 22—pyruvate dehydrogenase complex. **Vacuole**. Asp—aspartate; ALMT—aluminum$-$activated malate transporter; CAT—cationic amino acid transporter. **Cytosol**. Cit—citrate; Isocit—isocitrate; Pyr—pyruvate; GORK—guard cell outward rectifying K^+ channel; 17—SSR1; 23—aconitase; 24—isocitrate dehydrogenase; 25—glutamate:oxaloacetate (aspartate) transaminase or glutamate: pyruvate (alanine) transaminase; 28—PROT (proline transporter or GAT (GABA transporter).

Plastids and peroxisome are the main organelles in alternative GABA biosynthesis (Figure 1). In the peroxisome, putrescine, spermidine, and spermine are the major polyamines converted by FAD-dependent PA oxidases [23]. In further steps, ABAL is used to synthesize GABA from two aldehyde dehydrogenases [24]. In plastid, Put is produced from arginine by arginine decarboxylase, agmatine imidohydrolase, and carbamoylputrescine amidohydrolase, which is subsequently turned to ABAL. The glutamate–proline–GABA biosynthetic pathway is considered another alternative source of cytosolic GABA, which includes both enzymatic (such as NADP-dependent Δ1-pyrroline-5-carboxylate synthetase and NADP-dependent Δ1-pyrroline-5-carboxylate reductase) and non-enzymatic steps (such as proline spontaneous decarboxylation) [25]. However, currently, the significance of the proline involvement in the GABA production in plants has not been supported by direct evidence.

3. Physiological, Biochemical, and Molecular Aspects of GABA Metabolism in Plant Adaptation to Salt Stress

State-of-the-art omics technologies provide sensitive and precise methods for the global analysis of the development of salt stress tolerance in plants. Such methods include metabolomics, proteomics, and transcriptomics, which allow one to combine and analyze of complex relationships among metabolic networks, gene regulation, enzyme function, and specific metabolites [26,27]. Later in this section, we focus on recent publications describing the application of omics technologies to decipher the development of salt stress adaptation mechanisms in plants.

Plants have established a number of metabolic and developmental mechanisms to adapt to high soil salinity. Gradual increases in NaCl concentration in media cause enhanced salt tolerance in Arabidopsis root cells callus culture, which was maintained even after the stress factor was removed, suggesting some memory mechanism of acquired salt tolerance. Salt-stress-adapted cells have higher levels of sugars (sucrose, glucose, and fructose), amino acids (glutamine, valine, alanine, leucine, isoleucine, and threonine), shikimate pathway intermediates (tyrosine, coniferin, and flavanol glycosides), and fatty acids in comparison to control cells. However, the levels of TCA cycle intermediates (malate, succinate, citrate, and fumarate) were significantly lower in adapted cell lines. Most importantly, whereas most metabolites reverted to levels close to control cells, the amounts of sugars, alanine, acetate, and GABA further increase, suggesting their crucial role in salt stress adaptation and acquiring salt stress memory [28].

The analysis of salt-tolerance-associated metabolites of two halophytes (*Limonium albuferae* L. and *Limonium doufourii* L.) under salt stress conditions revealed a significant difference between these closely related species. *L. doufourii*, which is considered less salt-tolerant in comparison to *L. albuferae*, demonstrated a more emphasized degradation of photosynthetic pigments and an increased Na^+/K^+ ratio under salt stress. On the other hand, *L. albuferae* showed a higher accumulation of fructose and glucose and steady levels of malic and citric acids. Interestingly, both species strongly activate the glutamate pathway, which is associated with the subsequent accumulation of GABA and proline [29].

The comparison of the morphological, physiological, and biochemical changes in wild salt-tolerant barley *Hordeum maritimum* With. and salt-sensitive cultivar *Hordeum vulgare* L. cv. Lamsi after prolonged severe salt stress treatment (five weeks on 200 mM NaCl) revealed higher compartmentalization of sodium ions in the roots of halophytic barley species, which helped to preserve the photosynthetic apparatus. On the level of metabolites, salt-stressed *H. maritimum* demonstrated decreased levels of ornithine, asparagine, and minor amino acids, while GABA level increased. *H. vulgare* under salinity showed decreased levels of all amino acids, and the contents of GABA, glutamate, and ornithine were even lower than untreated controls. The removal of stress normalized levels of the majority of amino acids in *H. vulgare*, while levels of alanine, aspartate, GABA, glutamine, and minor amino acids were greatly increased in *H. maritimum*. Interestingly, the control plants of *H. vulgare* had

an overage higher than *H. maritimum* concentration of GB (glycine betaine), an important osmolyte, which was not much influenced by salinity stress [30].

Metabolic profiling of three invasive freshwater salt-sensitive species, *Elodea canadensis* Michx., *Myriophyllum aquaticum* (Vell.) Verdc., and *Ludwigia grandiflora* (Michx.) Greuter & Burdet, demonstrated different responses to salt stress, which rely on biosynthesis and/or accumulation of various compatible solutes and osmoprotectants. In *E. canadensis*, only the level of asparagine was increased almost 3-fold under salt stress conditions. *L. grandiflora* and *M. aquaticum* showed marked increases for almost all tested amino acids, with the most pronounced effect on tryptophane, arginine, asparagine, GABA, serine, alanine, threonine, and proline. Similarly, carbohydrates were poorly represented in *E. canadensis*, with only malate, fructose, sorbitol, and sucrose detected. Interestingly, in *M. aquaticum*, the abundance of four carbohydrates was mostly affected (sorbitol, gentobiose, myo-inositol, and sucrose), while in *L. grandiflora*, salt treatments greatly modified nearly all checked carbohydrates (with the least pronounced effect on fructose and malate) [31].

The comparison of salt-stress-related metabolites between salt-sensitive and salt-tolerant sorghum genotypes revealed an increased level of putrescine in the sensitive genotype, while the tolerant genotype showed increased levels of cadaverine, spermine, and spermidine. Additionally, the tolerant genotype had a higher accumulation of GABA, proline, free amino acids, and soluble sugars. The sensitive genotype, on the other hand, showed decreased activity of arginine decarboxylase and pronounce activation of polyamine oxidase, confirming different metabolic changes related to salinity stress adaptation in these two genotypes [32]. Similar differences to salt stress treatment were identified among salt stress-sensitive and -tolerant rice genotypes. As was shown, sensitive lines produced more catalase and peroxidase, while the level of superoxide dismutase was higher in tolerant lines. Additionally, levels of putrescine and glutamine were higher in sensitive lines, while tolerant lines produced more sucrose, acetic acid, and GABA [33]. Interestingly, opposite results were obtained in sugar beet roots (*Beta vulgaris* L.), where high salt treatment significantly reduced levels of amino acids (valine, isoleucine, methionine, glutamate, threonine, leucine, serine, and glycine), GABA, and glucose. On the contrary, levels of other amino acids (proline, tyrosine and tryptophan) and organic acids (isocitric and citric) were increased [34].

The salt-stress-associated function of the GABA shunt in woody plants is similar to that in other plants. Thus, NaCl treatment of poplar seedlings (*Populus alba* × *Populus glandulosa*) leads to activation of GAD, GABA-T, α-KGDH, and SDH enzymes and enhanced transcription of *GADs*, *GABA-Ts*, *SDHs*, *SCSs*, and *SSADHs* genes, which results in increased accumulation of GABA and glutamate. The content of soluble sugars and free amino acids was increased, while biosynthesis of succinate, malate, and citrate was nearly inhibited [35]. Similarly, a comparison of salt-sensitive and salt-tolerant tomato plants revealed an increased abundance of proteins involved in GABA, brassinolides, and starch biosynthesis (GABA-T, sterol side chain reductase, and chloroplastic/amyloplastic starch synthase) in salt-tolerant cultivars. Additionally, the activity and abundance of antioxidants (SOD, PPO (polyphenol oxidase), and POD) were higher in salt-tolerant cultivars [36].

3.1. The Involvement of GABA in the Different Stress Signaling Pathways

Calcium signaling plays an important role in GABA biosynthesis and accumulation. In particular, the application of exogenous Ca^{2+} to hypoxia-NaCl-stressed soy plants significantly enhances the expression and activity of GAD, which leads to increased GABA accumulation. On the other side, inhibition of GAD activity with ethylene glycol tetraacetic acid reduces GABA content. Under hypoxia-NaCl stress, polyamine degradation (via DAO, diamine oxidase) contributed over 31–39% of GABA formation, while specific DAO inhibition with aminoguanidine reduced GABA content by 33–36% in different organs [37]. Interestingly, experiments on fava beans (*Vicia faba* L.) under hypoxia-NaCl stress suggested that 43–81% of GABA have been synthesized via GABA shunt [38]. Furthermore, the interplay between GABA and Ca^{2+} signaling in lipid and ROS production were recently demon-

strated on microalgae *Monoraphidium* sp. QLY-1 under fulvic acid/salinity stress. Treatment of stressed algae with Ca^{2+} increased GABA content and expression of lipid biosynthesis-related genes (*accD* (acetyl-CoA carboxylase), *gpat* (glycerol-3-phosphoacyltransferase), and *dgat1* (diacylglycerol acetyl-transferase), thus improving algae stress resistance. Similarly, GABA treatment increased lipid production, transcription of the antioxidant *GSH* (glutathione) gene, and endogenous GABA production. These data suggest cross-talk between lipid, antioxidant, and GABA production and accumulation; Ca^{2+} signaling; and stress resistance in microalgae [39].

It has been recorded for barley that both glutamate and GABA could play a role in long-distance electrical signal transmission through plants [40]. Furthermore, intracellular Ca^{2+} signals modulated by glutamate-dependent Glutamate Receptor-Like family protein (GLR) might influence GAD activity and GABA signals [41–43].

The direct interaction of GABA with plasma membrane K^+ channel GORK could indicate participation in stress-induced K^+ leakage from the cells [16,44]. K^+ plays a dual role—nutritional and signaling [45,46]. Salinity stress is induced by K^+ loss in plants through GORK- and NSCC-type channels [46]. Furthermore, cytosolic K^+ efflux was proposed to play the role of the "metabolic switch" by providing inhibition of energy-consuming biosynthesis. The signaling model of the "metabolic switch" suggests the important role of cytosolic K^+ efflux in halting plant growth and using saved energy to finish a full life cycle [44,47]. Therefore, GABA could play a diverse and substantial role in regulating stress signaling in salt-stressed plants.

3.2. GABA Shunt and TCA cycle under Salt Stress

The exposures of wheat (*Triticum aestivum* L. var Wetsonia and Wyalkatchem) plants to salt stress resulted in a significant increase in proline, lysine, alanine, glutamate, and GABA accumulation. Interestingly, the levels of fructose, maltose, trehalose, and sucrose were increased, while the abundance of glucose was decreased. Among other metabolites, aconitate, citrate, malate, and fumarate showed a significant decrease under salt stress conditions, while the levels of succinate and 2-oxoglutarate were increased. Out of 38 TCA cycle proteins, the abundance of one isoform of succinate dehydrogenase subunit 2 was increased, while another isoform of succinate dehydrogenase subunit 2, aconitase, mtOGDC E2, and two isoforms of mtPDC E2 subunits were decreased after salt exposure. Interestingly, the abundance of pyruvate carriers (1 and 2) (VDAC 3 and TIM9) was decreased, while VDAC 1, a phosphate carrier, and a branched-chain amino acid carrier were increased. Specifically, to GABA shunt components, GDH and SSADH1 and -2 were increased, with no decreased proteins found. These data suggest that salt inhibited the main TCA cycle enzymes, while increased GABA shunt activity overcame these effects bypassing salt-sensitive enzymes and maintaining the flow of metabolites required for mitochondria [48]. Similarly, the application of combined high light and salinity stresses to durum wheat (*Triticum durum* Desf. cv. Ofanto) seedlings inhibited GB synthesis and decreased glutamate content, while levels of minor amino acids, amides, and GABA were increased, thus confirming their crucial role in metabolic adaptation to these combined stressors [49].

To sum up, salt stress impairs the normal functioning of the carbon utilization system in mitochondria; thus, instead of being used to produce sufficient amounts of ATPs via TCA, it would be spent on less efficient pathways. ATP is required for the synthesis of compatible solutes, with high amounts of antioxidants to match increased ROS levels and other metabolic processes, such as the enhanced operational rate of ion exclusion channels and transporters (primarily, for Na^+, K^+ and Ca^{2+}). However, the TCA cycle and GABA shunt enzymes and metabolites respond to salt stress differently in the leaves and roots of the same plant, suggesting tissue-specific variations. Additionally, the salt sensitivity background (tolerant or sensitive), the severity of salt stress and time of exposure, previous priming, nutrition supply status, and presence of other stresses (biotic or abiotic) would define the most affected enzymes and metabolites. Therefore, the represented example of the salt stress effect (Figure 2) should not be considered as a universal guideline. GABA

shunt activation during salt stress exposure allows the plant to overcome inhibited TCA cycle enzymes and maintain carbon–nitrogen balance. However, it is necessary to keep in mind that GABA shunt intermediates are involved in a number of interconnected metabolic processes, including cytosolic glycolysis, the oxidative pentose phosphate pathway, the mitochondrial TCA cycle, amino acids metabolism, and homeostasis, and many other pathways. Although certain metabolites and proteins involved in these processes and pathways were characterized a long time ago, their regulatory mechanisms and the impacts of salt exposure are still not well understood. Therefore, a thorough understanding of a metabolic network and its response to salinity is required for the successful implementation of GABA-based principles and methods in plant breeding.

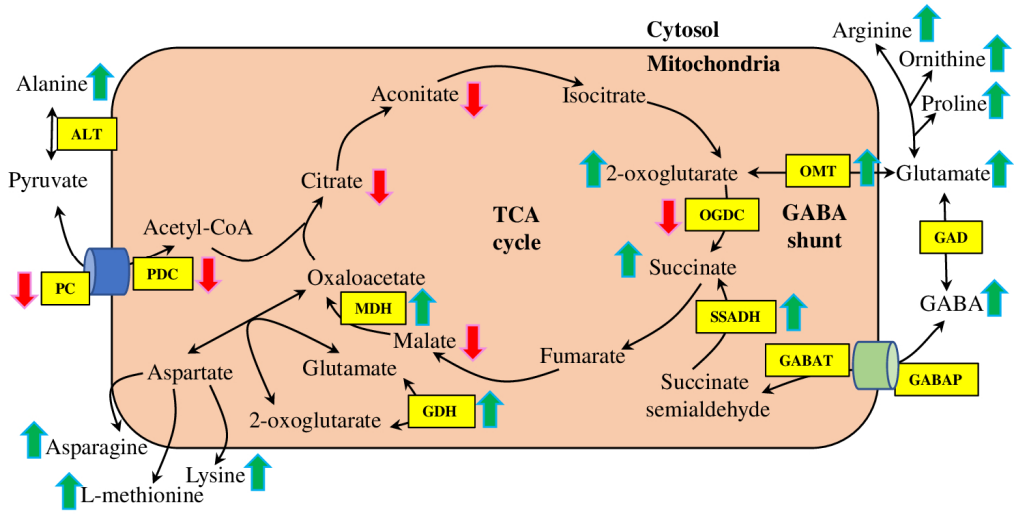

Figure 2. The effect of salt stress on TCA cycle and GABA shunt functioning. Salt stress impairs the pyruvate uptake by PC (pyruvate carrier) and PDC (pyruvate dehydrogenase), which leads to subsequent depletion of citrate and aconitate. Additionally, the level of OGDC (2-oxoglutarate dehydrogenase) decreases, thus decreasing 2-oxoglutarate conversion into succinate. However, the levels and activities of OMT (2-oxoglutarate dehydrogenase) and SSADH (succinate semialdehyde dehydrogenase) provide an alternative supply for the TCA cycle. In parallel, oxaloacetate and 2-oxoglutarate are used to produce surplus amounts of diverse amino acids. Abbreviations: GDH—glutamate dehydrogenase; MDH—malate dehydrogenase; ALT—alanine aminotransferase. Red arrows indicate decreased enzyme/metabolite activity/abundance, and green arrows indicate increased enzyme/metabolite activity/abundance.

4. Manipulation with Genes Involved in GABA Metabolism

Despite the great importance of GABA in plant response to environmental stresses, the exact molecular regulatory mechanisms behind GABA metabolism (GABA biosynthesis and catabolism) and transport are far less studied (mostly unknown). As was shown in tomato plants, the knock-down of both *GAD* and *GABA-T* results in increased salt sensitivity and increased ROS accumulation under salt stress conditions. However, GABA levels increased and decreased in the *GABA-T* and *GAD* mutants, respectively, while the level of succinate was decreased in both mutants. Interestingly, *SSADH* knock-down resulted in curled leaves, dwarf phenotype, and increased ROS accumulation in normal conditions, thus confirming the role of GABA shunt in the ROS metabolism through a bypath for succinic semialdehyde catabolism to γ-hydroxybutyrate and subsequent ROS accumulation under salt stress [50]. Similarly, the expression of mulberry tree (*Morus* L.) *GABA-T* gene in Arabidopsis plants and mulberry hairy roots leads to reduced GABA content, increased ROS accumulation, and salt stress sensitivity. However, GABA treatment alleviated ROS damage, increased the activities of antioxidant enzymes (SOD, POD, and CAT), and increased resistance to salt stress [51].

Recent research on *GABA-T* (*pop2*) and *GAD* (*gad1* and *gad2*) mutants has confirmed the role of GABA accumulation in salt stress tolerance. Therefore, *pop2* mutants accumulated more GABA and showed a salt-tolerant phenotype, while *gad1,2* double mutants were unable to obtain GABA from glutamate and have a salt-sensitive phenotype. Interestingly, the role of GABA in salt tolerance was associated with the expression and activity of various transporters and channels (H^+-ATPase, SOS1, NHX1, GORK channels), which normalized levels of Na^+ and K^+ ions, maintained membrane potential, and reduced H_2O_2 production [52]. However, the exact molecular mechanisms by which GABA levels control the expression and activity of ion transporters and channels are currently unknown and should be determined in future research.

Furthermore, the role of polyamines as a source of GABA was established in Arabidopsis cell suspension protoplasts transiently expressing *AtALDH10A8* and *AtALDH10A9* (aldehyde dehydrogenase) genes. ALDH accepts 4-aminobutanal and 3-aminopropanal as substrates and produces GABA and β-alanine, respectively. Additionally, single loss-of-function mutants were more sensitive to salt stress and accumulated less GABA [53]. *GATs* (GABA transporters) genes are responsible for primary GABA transport. The overexpression of *GAT* genes from poplar (*Populus euphratica* Oliv.) in Arabidopsis and poplar resulted in improved salt-stress tolerance via enhanced lignin content in xylem tissues and increased proline accumulation in poplar leaves [54].

In total, GABA accumulation via GABA shunt activation is crucial for stress responses, interaction with other metabolic pathways, and signaling functions. GABA accumulation is achieved via its increased biosynthesis, reduced catabolism, and/or enhanced transport. Additionally, manipulations with levels of GABA precursors could be used as GABA sources to improve salt stress tolerance.

5. Manipulation of Plant GABA Levels and Salt Stress Improvement by Exogenous Application of GABA or Its Precursors

In recent years, numerous studies have revealed that exogenously applied GABA has beneficial properties, protecting cells from oxidative damage, water loss, and photoinhibition and maintaining membrane integrity [55], thus facilitating adaptation to drought, high temperature, and salt stress in different plant species [56]. Later in this section, we focus on recent papers deciphering the molecular mechanisms activated by exogenous GABA application to enhance salt stress tolerance.

5.1. Phenolics Biosynthesis and Ca^{2+} Signaling

The basic mechanism of exogenous GABA effects was studied in citrus leaves (*Citrus* × *aurantiifolia* (Christm.) Swingle). Upon leaf treatment with GABA, levels of endogenous GABA, fumaric, and succinic acids were quickly increased, suggesting that the primary effect of GABA treatment relies on increased energy production via the TCA cycle [57]. These results were further confirmed with the application of deuterium-labeled D6-GABA, which had a half-life of about 1.3 h and was mostly translocated via xylem and quickly metabolized to succinic acid [58].

Recent research on hulled barley demonstrated the effect of exogenous GABA treatment on endogenous GABA metabolism and antioxidant capacity under salt stress. Therefore, GABA treatment increased the accumulation of endogenous GABA, and total amino acids content (the most drastic effect on glutamate, alanine, and proline). Additionally, increased activities of 4CL (4-coumarate coenzyme A ligase), C4H (cinnamic acid 4-hydroxylase), and PAL (phenylalanine ammonia lyase) resulted in higher content of total phenolic components and antioxidant capacity [59]. Interestingly, recent research demonstrated that a mild NaCl stress (1–20 mM) on barley seeds enhanced growth and PAL, C4H, and 4CL expression and activities, thus increasing the content phenolic and flavonoids (such as gallic acid, quercetin, fisetin, protocatechuic, and myricetin) and enhancing DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) free radical scavenging capacity [60]. However, further research showed that GABA

plays a major role in the accumulation of phenolics. Both mild salt stress and GABA treatment increased total phenolics and flavonoid content, PAL, C4H, and 4CL mRNA levels, and protein activities. Furthermore, the application of 3-MP (3-mercaptopropionic acid), the GABA biosynthesis inhibitor, reversed those beneficial effects (but increased PAL, C4H, and 4CL protein levels). These results suggest that low-level NaCl stress promotes plant growth and phenolic synthesis through stimulated endogenous GABA metabolism, which, subsequently, affects other key metabolic pathways in barley seedlings [61].

Similarly, the priming of rice plants with GABA alleviated the effect of salt and osmotic stresses through improved water relation parameters and photosynthetic systems, increased endogenous GABA, total sugars, protein and starch content, glutathione reductase, and K^+ concentration, while levels of malonaldehyde, free radical, and Na^+ were reduced. Interestingly, GABA-primed plants showed increased expression of *CIPK* (Calcineurin B-like Protein-interacting protein Kinases) genes (*CIPK01*, *CIPK02*, *CIPK03*, *CIPK07*, *CIPK08*, *CIPK09*, *CIPK12*, *CIPK15*, and *CIPK17*). These data indicated the close association between antioxidant enzymes, secondary metabolism, Ca^{2+} signaling, and the development of the adaptive stress tolerance [62]. Similar results were also obtained also for bentgrass (*Agrostis stolonifera* L.), where priming with GABA increased the total polyamines and spermidine content, sugars (talose, galactose, and maltose), and levels of amino acids involved in GABA shunt (GABA, alanine and glutamic acid) and other amino acids (glycine, aspartic acid, cysteine, threonine, serine, and phenylalanine), thus alleviating effect of abiotic stresses on chlorophyll content, osmotic potential, leaf relative water content, and other physiological parameters [63].

5.2. Photosynthesis and Antioxidant Defense

Photosynthetic apparatus and photosynthesis are two other targets that are greatly damaged by salt stress. Further in this section, we focus on recent papers which have addressed the beneficial effects of exogenous GABA application on various photosynthesis-associated functions.

The application of exogenous GABA on salt-stressed wheat seedlings increases the germination rate and shoot dry mass and decreases MDA content and REC (relative electrolyte conductivity), while enhancing antioxidant enzymes (SOD and CAT) activities, net photosynthesis rate, stomatal conductance, and transpiration and preventing the decline of pigment content Chlα (chlorophyll) and Chlβ [64]. Similarly, the application of GABA on moderately (150 mM NaCl) and severely (300 mM NaCl) stressed maize seedlings increased endogenous GABA concentration but decreased GAD activity. Furthermore, the levels of proline and soluble sugar were increased, and simultaneously, the contents of superoxide anion and MDA were decreased. Additionally, the levels of antioxidant enzymes (SOD, APX, CAT, and POD) were increased. The beneficial effect of GABA application was demonstrated on photosynthetic parameters (net photosynthetic rate, intercellular CO_2 concentration, and stomatal conductance) and chlorophyll fluorescence parameters (primary fluorescence, maximal fluorescence, maximum quantum efficiency of Photosystem II photochemistry, and electron transfer rate) [65]. GABA treatment was also beneficial for salt-stressed lettuce plants, where it improved photosynthetic performance and reduced oxidative damage. In particular, the application of GABA decreased electrolyte leakage, recovered decline in quenching coefficients and non-photochemical quenching, enhanced electron transport flux in photosynthetic apparatus, and improved the quantum yield of PS II (photosystem II). Additionally, the activities of antioxidant enzymes (CAT, SOD, and APX (L-ascorbate peroxidase)) were increased in GABA-exposed plants [66].

Similarly, GABA effectively improved the salt stress tolerance of broad beans (*Vicia faba* L.) in combination with melatonin (N-acetyl-5-methoxytryptamine). Both bioactive molecules increased the maximum quantum yield of PS II and minimized the negative effect of stress on the non-photochemical quenching and the energy fluxes of light absorption (Figure 3) [67]. Interesting molecular mechanisms of GABA-mediated effects on salinity–alkalinity-stressed muskmelon (*Cucumis melon* L.) plants have been described recently.

Priming with GABA significantly inhibited the effects of stress, such as reduced activities of glutathione reductase and monodehydroascorbate reductase, reduced content of GSH and AsA, and increased REC and MDA content. However, the ability of GABA to ameliorate stress-mediated effects was greatly reduced when the endogenous production of H_2O_2 was inhibited with diphenyleneiodonium, while the inhibition of AsA and GSH (with acriflavine and buthionine sulfoximine, respectively) had no such an effect. Considering the levels of chlorophyll synthesis precursors under salinity-alkalinity stress, exogenous GABA treatment increased the content of δ-aminolevulinic acid but reduced the contents of glutamate, porphobilinogen, uroporphyrinogen III, Mg-protoporphyrin IX, protoporphyrin IX, protochlorophyll, and chlorophyll [68].

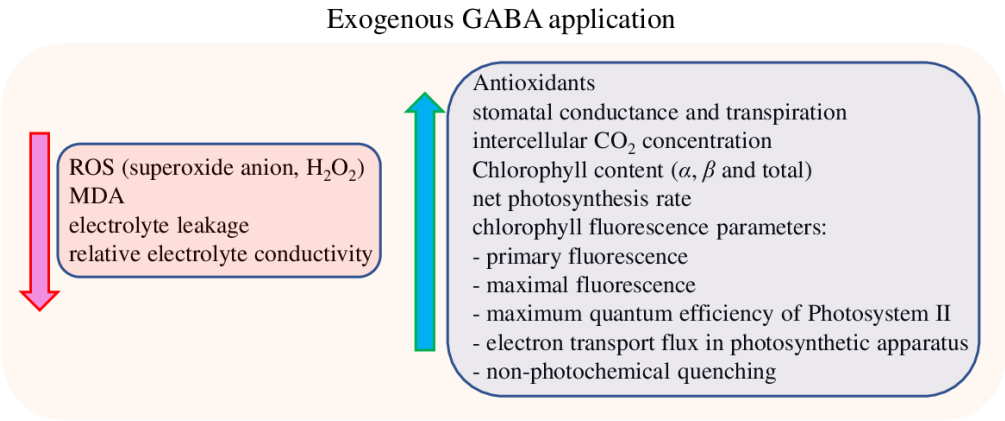

Figure 3. Chart summarizing the beneficial effects of GABA application on the photosynthetic apparatus, photosynthesis parameters, and antioxidant defense under salt stress.

Another mechanism of GABA-mediated salt-stress tolerance was investigated in the wheat plant. As expected, GABA treatment improved sulfur assimilation; nitrogen metabolism; Na^+ and K^+ ions homeostasis; photosynthesis and growth under salt; increased SOD, APX, and GR activities; and NO and proline content (Figure 3). However, the application of NO inhibitor c-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide) reversed the positive effects of GABA on growth, chlorophyll content, and net photosynthesis [69]. These results are consistent with the known role of NO in salt-stress response, further connecting salt stress, N and S assimilation, photosynthesis, and antioxidant systems [70].

5.3. Na^+/K^+ Ions Homeostasis

Recent research has demonstrated interesting results on salt-exposed maize seedlings. GABA treatment improved plant growth and chlorophyll content (*a*, *b*, and total chlorophyll); increased POD, CAT, APX, and SOD activities; and reduced oxidative damage. Additionally, the content of Na^+ was lower, and the content of K^+ was higher. However, the expression level of *HKT1* was increased, while the expression of *SOS1* and *NHX1* was decreased [71]. Similarly, GABA treatment increased growth parameters and chlorophyll content of salt-stressed tomato plants. Additionally, the expression of *GADs*, antioxidants (*SOD*, *CAT,* and *POD*) genes, and amino acid contents in tomato leaves were enhanced, while levels of superoxide anion, H_2O_2, and MDA were reduced. Interestingly, GABA prevented Na^+ influx in roots, thus preventing its transport to the leaves and accumulation, which causes further toxic effects on other systems [72].

Soaking white clover (*Trifolium repens* cv. Haifa) with GABA provided a wide range of positive effects on salt-stress tolerance. Increased expression of antioxidant genes and enzyme activities resulted in a decreased level of oxidative damage during germination. However, despite accelerated starch catabolism, the levels of free amino acid, free proline, and water-soluble carbohydrates were reduced. The expression and accumulation of dehydrins (*dehydrin b*, *Y2SK*, *SK2* and *Y2K*) were induced by exogenous GABA in seedlings

under salt stress. Furthermore, Na^+/K^+ content was improved by GABA treatment via increased the expression of several transporters and Na^+-toxicity-associated proteins (SOS1, H^+-ATPase, HKT1, HKT8, and HAL2) in salt-stressed seedlings (Figure 4) [73]. Interestingly, the application of Put (putrescine), one of the GABA precursors, has also been reported to provide a positive effect on salt tolerance in white clover. Therefore, soaking with Put significantly increased salt-stress tolerance and improved the growth parameters of seedlings and starch metabolism. The accumulation of many metabolites was increased by Put treatment, in addition to TCA cycle and GABA-shunt-associated metabolites, and levels of sugars (anhydro-D-galactose, fucose and levoglucosan), alcohols (threitol, hexadecanol and myo-inositol) lipids (linoleic, palmitic and stearic acids, glycerol), organic and amino acids (GABA, proline, glutamate, ketopentanic, malic and malonic acids), and polyamines (spermidine and Put) were increased. Additionally, the expression levels of transporters (*HKT1*, *HKT8*, *SOS1*, *SKOR* and *NHX6*) and detoxification-associated *HAL2* genes were increased [74]. Similar results have also been demonstrated with another GABA precursor spermine on bentgrass, suggesting the involvement of the same pathways and a rather universal method of activation for all GABA precursors [75].

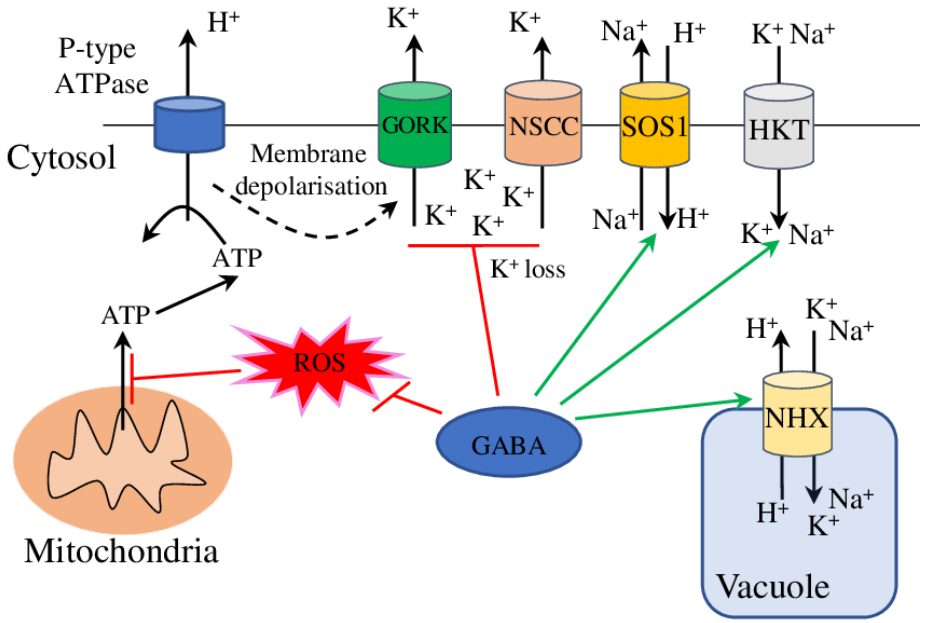

Figure 4. Integrative scheme of the role of GABA in the regulation of ion homeostasis. Red lines and green arrows represent the negative and positive effects, respectively, of GABA application on transporters and ion channels, their enzyme activity, and gene transcription.

Further clarification of the role of GABA in ions homeostasis was provided in a recent proteomic study on a salt-stress-sensitive variety of creeping bentgrass. GABA treatment upregulated the expression of *SOS1*, *NHX2/4/6*, *ATPaB2*, and *PPa2* in roots, but downregulated *HKT1/4* expression in leaves, thus maintaining higher K^+/Na^+ ratio, enhancing Na^+ compartmentalization, and mitigating Na^+ toxicity in the cytosol (Figure 4). Other pathways, activated by GABA treatment and facilitating salt tolerance, rely on enhanced sugar and amino acid accumulation, antioxidant defense, and increased accumulation of myo-inositol and heat shock proteins [56].

5.4. Phytohormones

Recent results have suggested that among various metabolites, amino acids, sugars, and endogenous GABA upregulated by GABA treatment, exogenous GABA application also increased the levels of most phytohormones via upregulated expression of their biosynthetic genes. As was shown on citrus (*Citrus* × *sinensis* (L.) Osbeck) GABA-treated plants, the upregulated expression of *GABA-T* and *SSADH* was accompanied by increased ac-

cumulation of glycine, L-alanine, L-proline, L-glutamine, L-asparagine, and TCA-cycle enzymes (malate dehydrogenase and succinic dehydrogenase). Most importantly, the genes responsible for the biosynthesis of phytohormones were upregulated, which subsequently led to increased levels of indole acetic acid, trans-jasmonic acid, benzoic acid, indole propionic acid, cinnamic acid, abscisic acid, and salicylic acid in GABA-treated plants compared to the control [76]. Furthermore, the closest association has been identified between GABA-mediated salt-stress tolerance and ethylene and abscisic acid signaling pathways. Thus, transcriptomic analysis of salt-stressed *Populus tomentosa* Carr. plantlets after GABA treatment revealed the involvement of many genes related to ABA metabolism (*ABAH2*, *ABAH4* and *ABAG*) and receptors (*PYL1/2/4/6*) and ethylene biosynthesis (*ACO1/2/5* and *ACS1/7*) (Figure 5) [77].

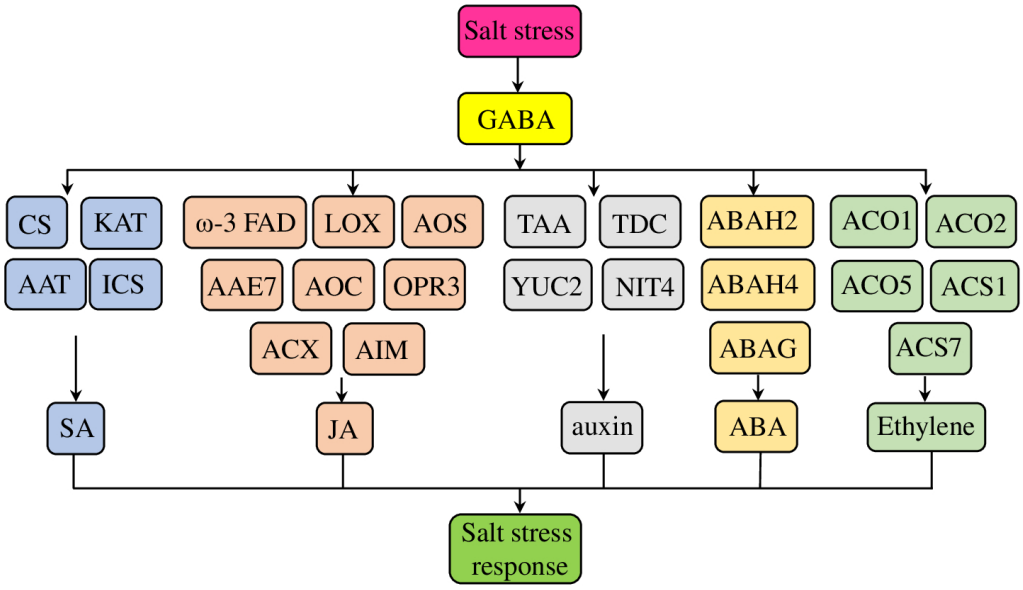

Figure 5. Proposed model of the cross-talk between salt stress, GABA, selected phytohormones biosynthesis genes, and salt stress response. SA (salicylic acid) biosynthetic genes: CS—chorismate synthase, ICS—isochorismate synthase, KAT—3-ketoacyl-CoA thiolase, AAT—alcohol acyl transferase; tJA (trans-jasmonic acid): ω-3 FAD—ω-3 fatty acid desaturase, LOX—lipoxygenase, AOS—allene oxide synthase, AOC—allene oxide cyclase, OPR3—12-oxophytodienoate reductase 3, AAE7—acetate/butyrate–CoA ligase AAE, ACX—acyl-coenzyme A, AIM—enoyl-CoA hydratase; auxin: TAA—tryptophan aminotransferase-related protein 2-like, TDC—tyrosine decarboxylase, YUC2—indole-3-pyruvate monooxygenase YUCCA2, NIT4—bifunctional nitrilase/nitrile hydratase NIT4A-like; ABA (abscisic acid): ABAH—abscisic acid 8-hydroxylase, ABAG—abscisate beta-glucosyltransferase; Ethylene: ACO—1-Aminocyclopropane-1-carboxylate oxidase, ACS—1-Aminocyclopropene-1-carboxylate synthase.

These results indicate that GABA functions in harmony and close association with phytohormones and suggest that GABA could facilitate the development of salt stress tolerance via the regulation of phytohormone signaling and biosynthesis.

Overall, the application of exogenous GABA is a promising strategy to enhance GABA shunt activity and increase endogenous GABA levels, which would reinforce carbon flux and elevate energy production. Adequate energy production during salt stress exposure is crucial for (1) the proper function of antioxidant (scavenging) and ROS-avoiding systems; (2) driving the production and accumulation of polyamines and proline; (3) activating secondary radical-scavenging pathways (such as anthocyanins, flavonoids, and phenols); (4) maintaining membrane stability and fluidity; (5) protecting the photosynthesis apparatus; and (6) supporting enhanced activity of ion channels and transporters to maintain ions homeostasis.

6. Conclusions

Soil salinization is a global problem, and its solution requires the application of multiple research approaches that would facilitate and enhance crop growth and yield. Currently used approaches rely on the metabolic pathways affected by salinity and the metabolic engineering of these pathways to maintain and enhance energy harvesting and production by photosynthesis and mitochondria, respectively. In this review, we focused on GABA as the central hub metabolite, connecting different pathways impaired by salt stress and providing an alternative metabolic pathway (GABA shunt) to increase energy production and alleviate the negative effects of salt stress (Figure 6). The wide application of modern "omics" methods has allowed the identification of many pathways and metabolites affected by salt stress; however, the characterization of corresponding enzymes inhibited by salinity is still missing. Additionally, the described metabolites (sugars, amino acids, antioxidants, etc.) and metabolic pathways (GABA-shunt) were examined individually, while their holistic evaluation is yet to be completed. Existing data suggest that some enzymes are more sensitive to salt stress exposure than others, blocking the entire pathway or cycle. However, the identification and implementation of salt-tolerant isoforms of these enzymes into breeding and genetic engineering programs requires a deep knowledge and understanding of the molecular mechanisms of their salt-mediated inhibition and GABA-mediated stimulation.

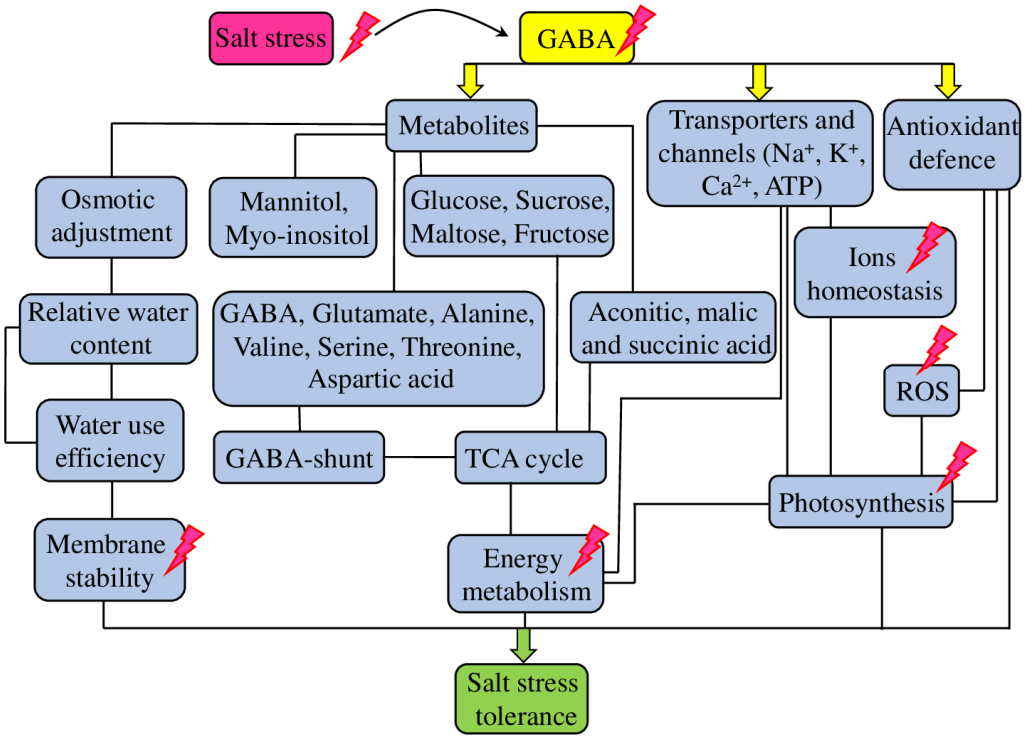

Figure 6. Different metabolites and signaling pathways activated by GABA to mitigate salt stress in plants (yellow arrows). The primary targets of salt stress are represented by magenta lightning.

The beneficial effect of GABA treatment and the role of GABA shunt activation under salt stress are well documented, while the exact molecular mechanism for this process is yet to be revealed. Most likely, GABA performs a dual function, acting as both an energy-providing metabolite and a carbon source to feed an alternative route for the TCA cycle during salt stress exposure. At the same time, the results of many papers presented in this review are hard to interpret because the origin of many metabolites (sugars and amino and organic acids) is difficult to define without specific metabolic tags. Therefore, the application of radiolabeled tracers would help to explore salt-affected and GABA-activated metabolic pathways, to define the difference between salt-tolerant and salt-sensitive varieties, and to

find the more promising target for breeding and/or metabolic engineering. On the negative side, surplus production of compatible solutes and amino acids during salt stress could originate from the degradation of other biomolecules; thus, it could be a disadvantage and affect final crop yield, quantity, and quality.

As another point to consider, the effect of salt stress and GABA treatment should be studied as a metabolic network with wide variation in germplasm (genetic diversity on the intra-specific and inter-specific levels) with diverse interaction between different pathways on multiple levels and actual salinity environments, which usually greatly differ from salt stress conditions modulated in phytotrones and greenhouses and often combine other biotic and abiotic stresses.

Future efforts toward the understanding and comparison of the metabolites flux and networks analysis on different organs, multi-tissue, and multi-cell levels will provide important data to create reliable computational models of the effects of the salt stress on plants' growth and development and are likely to facilitate the improvement in crops' yield and quality. Additionally, future applications of omics technologies and computational modeling will help to understand the role of particular metabolites in the regulation of individual genes and allow advanced plant breeding to achieve salt tolerance.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae9020230/s1. Figure S1: Simplified model of *Arabidopsis thaliana* GABA metabolism and signaling. The grey ovals and red rectangles represent important metabolites and enzymes (GABA-T (GABA transaminase), GABP (GABA permease), and SSADH (succinic semialdehyde dehydrogenase), GAD (glutamate decarboxylase), respectively, in the GABA shunt. The yellow squares represent transporters linking the TCA cycle back to the GABA shunt, and the small blue squares represent transporters linking the GABA shunt to other metabolic pathways and organelles (DIT2 and CAT9). The big blue square represents GABA-transporting ALTM1. Abbreviations: GABA, $\gamma-$aminobutyrate. **Peroxisome**. Spm—spermine; Spd—spermidine; Put—putrescine; ABAL—4-aminobutanal. **Plastid**. Glu—glutamate; Pro—proline; Arg—arginine. **Mitochondria**. GABA−T, GABA transaminase; Ac−CoA—acetyl−CoA; 2−OG—2−oxoglutarate; OAA—oxaloacetate; DTC—dicarboxylate/tricarboxylate carrier; UCP1—uncoupling protein 1. **Vacuole**. CAT—cationic amino acid transporter. **Cytosol**. Cit—citrate.

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