Chapter 12
GABA and Proline Metabolism in Response to Stress

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Abstract Environmental and biotic stresses induce metabolic changes in plants which led to the accumulation of specific metabolites. For instance, the accumulation of gamma-aminobutyric acid (GABA) and proline is a conserved response of plants to a wide range of stresses. In particular, these amino acids accumulate the most under abiotic stress conditions and, to a lesser extent, under biotic stress conditions. Multiple shared roles have been assigned to these molecules, including osmoprotection, osmotic adjustment, carbon source, redox balance and antioxidant functions. For some of these roles, however, the physiological function of these molecules has been a matter of debate. Recent studies point to unexplored functions of these molecules. For instance, proline accumulation was suggested to contribute to sustaining photosynthesis under stress conditions and proline catabolism was

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D. K. Gupta, F. J. Corpas (eds.), *Hormones and Plant Response*, Plant in
Challenging Environments 2, https://doi.org/10.1007/978-3-030-77477-6_12

suggested to be relevant to induce plant autophagy. Moreover, many enzymes of proline metabolism were suggested to be important for optimal response to biotic stress. This is even clearer for GABA, for which several mechanisms of action were already described to explain its capacity to protect the plant against pathogens. However, the role of GABA under biotic stress is highly dependent on the biotic stressor involved. Furthermore, GABA was recently demonstrated to enhance biotic stress tolerance when applied as a priming agent and its effect seems to be dependent on ethylene. Here we will summarize and integrate the current knowledge of GABA and proline accumulation in response to stress and discuss future perspectives.

Keywords Gamma-aminobutyric acid · Osmoprotection · Proline · Redox control · Autophagy

12.1 Introduction

Proline and **γ**-amino butyric acid (GABA) are amino acids present in plant cells sharing a relevant similarity: both have been extensively associated with stress responses in plants. Because of that, the induction of their metabolism is considered nowadays as reliable stress marker in plants. Although, their main functions under stress conditions is not completely understood. Another common characteristic of these amino acids is that the main biosynthetic pathway for each of them is from glutamate. Despite these similitudes, these amino acids are structurally different and while proline is a proteogenic amino acid, GABA is not. Moreover, they tend to accumulate in different types of leaves under non-stressed conditions. For instance, the concentration of GABA tends to be higher in old leaves than in young leaves, whereas proline concentration seems to be higher in young leaves compared to old leaves (Masclaux et al. 2000; Martínez et al. 2005; Dellero et al. 2020).

In this chapter, we describe the main biosynthetic mechanism and metabolic pathways in which these amino acids are involved. Next, we revise the literature showing their association with abiotic and biotic stress and discuss their potential functions in such conditions, which spans from energy fuelling for attacked plant cells, control of Reactive Oxygen Species (ROS) homeostasis, osmoprotection and interference of neurotransmission within herbivory predators to potential signalling roles, including possible cross-talk with phytohormones signalling pathways. Finally, we discuss the upcoming challenges to understanding the contributions of proline and GABA to stress tolerance in plants.

12.2 Biosynthesis and Degradation of GABA in Plants

In plants, GABA is synthesized from glutamate and Δ^1-pyrroline. The synthesis of GABA from glutamate via Glutamate Decarboxylase (GAD) is considered the pathway contributing the most to GABA production in plants (Shelp et al. 1999). This was demonstrated to be the case at least in roots, where the mutation of the root-specific GAD1 resulted in a dramatical decrease of GABA levels (Bouché et al. 2004). In this one-reaction pathway, a proton (H^+) is incorporated into glutamate which is decarboxylated to yield GABA (Fig. 12.1). Of note, this reaction consumes H^+ and it is believed that under important GABA accumulation, the H^+ consumption can be result in significant changes of pH and membrane potential due to changes in proton gradients (Crawford et al. 1994; Wegner and Shabala 2019). An alternative mechanism is the biosynthesis of GABA from Δ^1-pyrroline which can be converted into GABA thorough the action of Δ^1-pyrroline Dehydrogenase (PYRR-DH, Flores and Filner 1985). Proline and polyamines (PA) can contribute to the biosynthesis of Δ^1-pyrroline by different pathways leading to an increase of GABA (Figs. 12.1 and 12.3). In particular, proline was suggested to contributes to Δ^1-pyrroline through proline decarboxylation produced by the scavenging of hydroxyl radicals (·OH) (Signorelli et al. 2015). Whereas the polyamine spermidine and its diamine precursor, putrescine, are catabolized through the activity of Polyamine Oxidase and Diamine Oxidase to yield respectively (i) Δ^1-pyrroline and 1,3-diaminopropane, and (ii) ammonia, hydrogen peroxide and 4-aminobutanal, which spontaneously

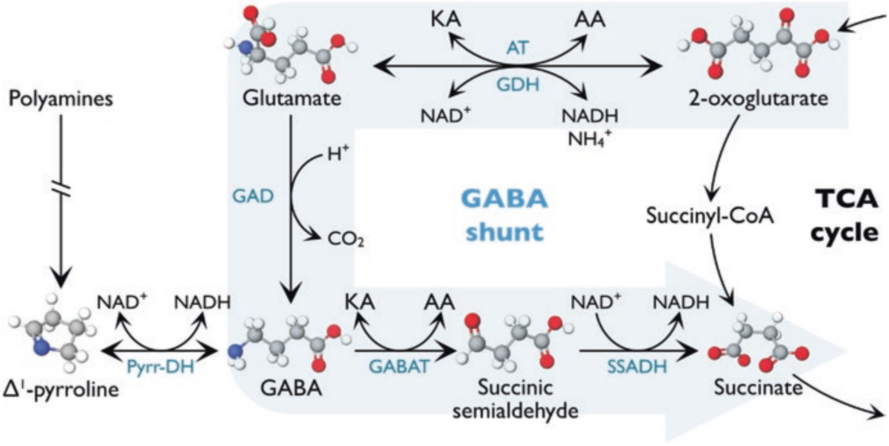

Fig. 12.1 GABA shunt and related metabolic pathways. The light blue arrow indicates the reactions involved in the GABA shunt. GABA shunts refer to the by-pass of two reactions of the tricarboxylic acid (TCA) cycle by alternative reactions involving GABA. Therefore, GABA shunts include reactions of GABA biosynthesis and degradation. Note that the TCA cycle is represented counter clockwise. *AA* amino acids, *AT* amino transferases, *GAD* glutamate decarboxylase, *GABAT* GABA transaminases, *GDH* glutamate dehydrogenase, *KA* keto acids, *SSADH* succinic semialdehyde dehydrogenase, *Pyrr-DH* Δ^1-pyrroline dehydrogenase

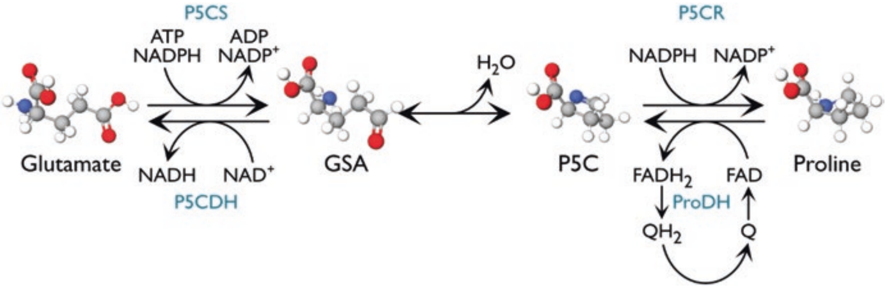

Fig. 12.2 Proline biosynthesis from glutamate and its catabolism. *GSA* Glutamate-5-semialdehyde, *P5C* Δ^1-pyrroline 5-carboxylate, *P5CDH* P5C Dehydrogenase, *P5CR* P5C reductase, *P5CS* P5C synthase, *ProDH* Proline Dehydrogenase, *QH2/Q* represents the mitochondrial respiratory chain

cyclizes to Δ^1-pyrroline (Fait et al. 2011; Shelp et al. 2012). These enzymes have been determined to be cytosolic and peroxisomal, indicating that peroxisomes contribute to GABA production through the PA pathway (Corpas et al. 2019).

GABA degradation consists of the elimination of the amine group and the degradation of the carbons through the tricarboxylic acid (TCA) cycle. Thus, the first reaction of GABA catabolism is the transamination of GABA mediated by GABA Transaminases (GABAT) to yield succinic semialdehyde (SSA). In this reaction the amine of GABA is transferred to another ketoacid such as 2-oxoglutarate, pyruvate or glyoxylate, to produce the respective amino acid (glutamate, alanine or glycine respectively). Different GABAT will use different keto-acids and this varies between plant species (Trobacher et al. 2013). The resulting SSA can be oxidized to succinate by the NAD$^+$ dependent Succinic Semialdehyde Dehydrogenase (SSADH) (Busch and Fromm 1999), with the formation of NADH, and the resulting succinate is catabolized into the TCA cycle. Alternatively, SSA can be converted to γ-hydroxybutyrate by an NADPH-dependent Glyoxylate/SSA Reductase (Fait et al. 2005).

12.3 Proline Metabolism in Plants

Proline is also produced mainly from glutamate, but after three reactions, two of them catalysed enzymatically and a spontaneous reaction. The first reaction is a two-step reaction and consists in the conversion of glutamate to glutamate-5-semialdehyde (GSA, Fig. 12.2). This reaction is catalysed by Pyrroline-5-carboxylate Synthase (P5CS), which consumes ATP in the first step of the reaction to produce the intermediate γ-glutamyl γ-phosphate, and NADPH in the second step (Hu et al. 1992). In most plant species two P5CS isoforms were identified, being one of them (usually named P5CS1 or P5CSA) inducible to stress conditions, and the other one constitutive (usually named P5CS2 or P5CSB) (Signorelli and Monza 2017). These isoforms were also show to express in differentially in diverse tissues and play differential roles (Szekely et al. 2008). The resulting GSA produced by P5CS is spontaneously cycled to produce 1-pyrroline-5-carboxylate (P5C), representing the second reaction of this biosynthetic pathway (Fig. 12.2). The third reaction is the reduction of P5C into proline catalysed by Pyrroline-5-carboxylate Reductase (P5CR) with the preferable consumption of NADPH (Giberti et al. 2014). Other enzymes such as Ornithine Cyclodeaminase and Pyrroline-2-carboxylate Reductase are involved in proline biosynthesis in prokaryotes but they were either not identified in plants or are not functional (Sharma et al. 2013), thus, in plants, P5C can be considered the sole precursor for proline biosynthesis (Trovato et al. 2019). Proline biosynthesis is known to take place in the cytoplasm, but it is also suggested to occur in the chloroplast under stress conditions (Szekely et al. 2008). However, more evidence is necessary to confirm the potential chloroplastic biosynthesis.

Proline catabolism consists of its conversion into glutamate in the mitochondria, involving the same intermediates of its biosynthesis, but in this case, the enzymatic reactions are catalized by Proline Dehydrogenase (ProDH, also known as Proline Oxidase, POX) and Pyrroline-5-carboxylate Dehydrogenase (P5CDH). Opposite to its biosynthesis, in these reactions reducing power is produced as it is an oxidative pathway (Fig. 12.2). In particular, ProDH is localized at the inner mitochondrial membrane and uses $FADH_2$ as co-factor, transferring the electrons directly to the mitochondrial respiratory chain.

Nowadays, we know that the P5CS1 promoter contains many cis-responsive elements such as ABF, AP2/EREBP, ERF2, DREB/CBF and MYB binding sites (Fichman et al. 2015; Zarattini and Forlani 2017). The presence of ABF explains why proline is accumulated in osmotic and saline stresses where ABA is produced. Likewise, proline accumulation was shown to be induced by light, being Elongated Hypocotyl 5 able to bind G-box and C-box elements of P5CS1 (Feng et al. 2016; Kovács et al. 2019). In addition, the Respiratory Burst Oxidase Homolog-dependant hydrogen peroxide was reported to stimulate proline accumulation in plants by increasing P5CS activity and potentially decreasing ProDH activity in arabidopsis and wheat seedlings (Ben Rejeb et al. 2015; Liu et al. 2020). Other factors affecting proline metabolism include the age of the tissue. For instance, younger (sink) leaves accumulate more proline and degrade it less, whereas older (source) leaves accumulate less proline and degrade more (Dellero et al. 2020).

12.4 GABA and Proline Involvement in Abiotic Stresses Responses

Both GABA and proline have been reported to accumulate in many plant species under diverse abiotic stress conditions (Table 12.1). GABA was initially considered to accumulate in response to diverse stresses such as hypoxia, wounding, drought, heat or cold stress (Shelp et al. 1999), but in many other cases reductions in GABA

Table 12.1 Changes in proline and GABA content are a common response of a variety of plants to diverse abiotic stress treatments

Stress	Plants	Response		References
		Proline	GABA	
Cold treatment	Loquat fruit	+	+	Cao et al. (2012)
	Mulberry	ND	+	Li et al. (2018a)
	Wheat seedlings	+	+	Naidu et al. (1991)
	Date palm tree	+	ND	Yaish (2015)
Water deficit	Potato plants	+	ND	Büssis and Heineke (1998)
	Maize (root apex)	+	+	Verslues and Sharp (1999)
	Eucalyptus	ND	+	Otto et al. (2017)
	Sesame	ND	+	Bor et al. (2009)
	Cowpea	+	ND	Zegaoui et al. (2017)
	Date palm tree	+	ND	Yaish (2015)
	Lotus uliginosus	+	+	Díaz et al. (2005)
Desiccation	Arabidopsis	+	ND	Yoshiba et al. (1995)
Osmotic stress	Arabidopsis	+	–	Chiang and Dandekar (1995)
Heat stress	*Lotus corniculatus*	+	ND	Signorelli et al. (2013a, b)
	Red clover	+	ND	Signorelli et al. (2013a, b)
	Mung bean	ND	–	Priya et al. (2019)
	Lentil	+	ND	Sita et al. (2018)
	Sesame	ND	+	Bor et al. (2009)
	Date palm tree	+	ND	Yaish (2015)
Saline	Arabidopsis	+	–	Chiang and Dandekar (1995)
	Alfalfa	+	ND	Miller et al. (2005)
	Arabidopsis	+	ND	Verbruggen et al. (1993)
	Barley	+	ND	Fedina et al. (2002)
Selenium stress	Sesame	ND	+	Bor et al. (2009)
UV	Rice	+	ND	Saradhi et al. (1995)
	Mustard	+	ND	Saradhi et al. (1995)
	Mung bean	+	ND	Saradhi et al. (1995)
Fe deficiency	Cucumber	ND	+	Guo et al. (2020)

(+) indicates increase; (–) indicates reduction; *ND* No data

have been reported as well (Table 12.1), which usually is interpreted as a mechanism to provide the necessary energy to cope with the stress condition through the GABA shunt. Other authors have reported that the regulation of the GABA shunt is the key response, independently of the GABA levels. This is the case for two *Brachypodium sylvaticum* cultivars with contrasting tolerance to freezing, which showed the same concentration of accumulated GABA under stress condition, but differential regulation of GABA shunt (Toubiana et al. 2020). The most tolerant cultivar downregulated the GABA shunt activity whereas the most sensitive upregulated it, and the authors concluded that the downregulation by the tolerant cultivar saved resources for the fatty acid biosynthesis displaying a better membrane integrity than the sensitive cultivar (Toubiana et al. 2020).

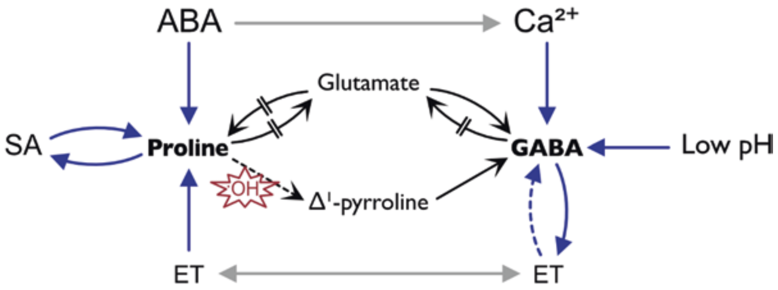

Fig. 12.3 Links between proline and GABA metabolism and plant signals. The abbreviations of the hormones are as described in the main text. The size of the font of the hormones related to how well document is the potential link. Blue lines indicate signals having an effect of proline or GABA metabolism; grey lines indicate link between signals; black lines describe metabolic pathways; broken lines indicate several steps in a metabolic pathway; and dashed lines indicate processes not fully proven *in vivo*

Different known mechanisms induce GABA accumulation under stress conditions. One of them relates to the drops in pH produced under certain stress conditions because lower pH activates GAD resulting in higher GABA accumulation (Crawford et al. 1994). Another mechanism involves calcium (Ca^{2+}) as the trigger to induce GABA accumulation because the plant GAD was demonstrated to have a calmodulin-binding domain meaning that Ca^{2+} can modulate GAD activity through calmodulin (Baum et al. 1993). As many stresses cause a rapid increase of cytosolic Ca^{2+} the calmodulin-dependent GAD activation seems to a be major mechanism controlling GABA accumulation under stress.

Proline is also accumulated in response to stress, but its accumulation seems to rely more on the presence of phytohormones (further elaborated in Sect. 12.8) rather than Ca^{2+} concentrations or pH changes. This, in part, explains why proline accumulation is a slower response when compared to GABA. For instance, in soybean plants subjected to drought stress, proline accumulation occurs far after other physiological parameters such as stomatal conductance or leaf hydric potential are affected by the stress condition (Casaretto et al. 2021).

Abiotic stress conditions are often associated with the establishment of oxidative stress (Corpas et al. 2013). Under such conditions, the scavenging of hydroxyl radicals by proline, was determined by computational chemistry to be prone to produce proline decarboxylation, leading to the production of the GABA precursor Δ^1-pyrroline (Fig. 12.3) (Signorelli et al. 2015), providing another metabolic link between these molecules that often accumulate together.

Another response to abiotic stress is the increase of autophagic activity, which was shown to be important for abiotic stress tolerance (Liu et al. 2009). In this context, the accumulation of both GABA and proline was proposed to contribute to the osmotic adjustment required during autophagy to avoid mega-autophagy (Signorelli et al. 2019). This would mean that not only their metabolism, but also the molecules itself contribute to stress tolerance. In this sense, it is worth mentioning than many works have used exogenous application of GABA and proline during stress conditions showing positive effects (Islam et al. 2009; Song et al. 2010; Kaushal et al. 2011; Nayyar et al. 2014; Li et al. 2016). In these works, usually, the beneficial effects were attributed to the osmotic adjustment or their capacity to enhance antioxidant defence (discussed further in Sects. 12.6 and 12.7 for each molecule independently). Moreover, Rodríguez-Ruiz et al. (2019) showed that roots of pea plants exposed to arsenate increased the endogenous levels of proline and GABA but not leaves, where arsenic was poorly accumulated. This not only shows how proline and GABA respond according to the extent of the stress in different tissues but also that their accumulation is not a systemic response and it is rather localized.

Summarizing, in general, GABA is rapidly and transiently accumulated by stress factors but then degraded during prolonged stress. Whereas in the case of proline, most of the evidence shows a cumulative increase of proline levels throughout the stress and the accumulation does not stop until the stress condition is released. This statement is true if comparing proline accumulation along the different days of the stress treatments at the very same time of the day, because proline accumulations follow a diurnal cycle, resulting in the transient reduction of proline levels at nights even during a stress situation (Sanada et al. 1995; Hayashi et al. 2000).

12.5 GABA and Proline Responses in Plants Under Biotic Stresses

Changes in GABA and proline metabolism have also been observed in response to biotic stressors in different plants (Table 12.2). In the case of GABA, its accumulation following wounding was already reported in the 80s (Wallace et al. 1984), and further research in the 90s showed that such phenomenon takes place following herbivore invasion as well (Ramputh and Bown 1996). The authors observed that such endogenous GABA increase leads to growth inhibition and decreases survival rates of oblique-banded leaf roller (*Choristoneura rosaceana*) larvae. Subsequent research lines showed that GABA accumulation takes place following infection by fungi and bacteria as well (Seifi et al. 2013; O’Leary et al. 2016; Wang et al. 2019). A typical event occurring after recognition of many pathogens is an increase of $[Ca^{+2}]_{cyt}$ (Seybold et al. 2014) a condition that stimulates the activity of GAD through calmodulin (Snedden et al. 1995) leading to GABA accumulation, which fuels the GABA shunt in the mitochondria (Fait et al. (2005); Fig. 12.1). The GABA shunt activation is thought to be critical to containing a pathogen’s advance by supplying energy to attacked cells and modulating the Hypersensitive Response (HR), a strong immune response aimed at limiting a pathogen’s spread by creating an unfavourable environment for pathogen proliferation through biochemical events such as localized ROS accumulation (Kim et al. 2013; Seifi et al. 2013; Tarkowski et al. 2019). Regarding the first feature, it was demonstrated that in the tomato *sitiens* mutants unable to accumulate ABA, overaction of the GS/GOGAT cycle together with enhanced expression and enzyme activity of the GABA shunt genes

Table 12.2 Different pathosystems in which GABA or proline metabolism were shown to be activated

	Pathogen/pest	Lifestyle	Host	Tissue	References
GABA	*Agrobacterium tumefaciens*	Biotroph	*Arabidopsis thaliana*	Crown gall tumor	Lang et al. (2016)
	Botrytis cinerea	Necrotroph	*Lactuca sativa*	Leaf	Tarkowski et al. (2019)
	Choristoneura rosaceana	Herbivore	*Glycine max*	Leaf	Ramputh and Bown (1996)
	Cladosporum fulvum	Biotroph	*Solanum lycopersicon*	Leaf	Solomon and Oliver (2002)
	Magnaporthe oryzae	Hemibiotroph	*Oryza sativa*	Cell suspension	Forlani et al. (2014)
	Pseudomonas syringae	Hemibiotroph	*Phaseolus vulgaris*	Leaf	O'Leary et al. (2016)
	Rhizoctonia solani	Necrotroph	*Glycine max*	Leaf	Copley et al. (2017)
	Spodoptera littoralis	Herbivore	*A. thaliana*	Leaf	Scholz et al. (2017)
Proline	*Melampsora lini*	Biotroph	*Linum usitatissimum*	Leaf	Ayliffe et al. (2002)
	P. Syringae	Hemibiotroph	*A. thaliana*	Leaf	Fabro et al. (2004)
	P. Syringae	Hemibiotroph	*Nicotiana benthamiana*	Leaf	Senthil-Kumar and Mysore (2012)
	Rhizoctonia cerealis	Necrotroph	*Triticum aestivum*	Leaf	Zhu et al. (2014)
	Mayetiola destructor	Herbivore	*Triticum aestivum*	Leaf	Zhu et al. (2008)

GABAT and SSADH positively correlates with resistance to the necrotrophic fungus *Botrytis cinerea*, while application of the GABA shunt inhibitor HBA (4-hydroxybenzaldehyde) abolished the resistant phenotype (Seifi et al. 2013). Consistently, enhanced TCA cycle and GABA shunt activities were shown through a metabolomic approach to be closely associated with resistance to the fungus *Fusarium graminearum* in Arabidopsis (Chen et al. 2018), pointing toward a critical role for GABA metabolism in immune responses against fungal pathogens (Tarkowski et al. 2019). However, GABA accumulation can also be detrimental for the host in the case of bacterial pathogens, which are mostly characterized by biotrophic and hemibiotrophic lifestyles (meaning that they need to keep host cells alive to be able to feed on them), in contrast with the necrotrophic lifestyle of most fungal pathogens (Dean et al. 2012; Ökmen and Doehlemann 2014). This happens because some bacterial pathogens were shown to be able to take advantage of GABA accumulation in the apoplastic environment by using it as energy sources themselves (Solomon and Oliver 2002; Rico and Preston 2008). Further research in this sense showed that GABA is utilized by the bacterial pathogen *Pseudomonas* *syringae* only when other preferred C and N sources are depleted, thus highlighting the adaptation of this pathogen to host metabolism (McCraw et al. 2016; O'Leary et al. 2016).

Interestingly, insect-triggered GABA accumulation looks to be dependent on different mechanisms compared to pathogens. A recent study showed that this event does not depend on $[Ca^{+2}]_{cyt}$ fluctuations, thus excluding GAD activity as a source of GABA (Scholz et al. 2017). What triggers GABA accumulation in such conditions remains to be understood.

Regarding proline, Ayliffe et al. (2002) found that flax plants accumulate transcript of a gene (*FIS1*) at the rust infection sites, and this gene turned out to encode for a P5CDH, suggesting that that proline catabolism played a role in plant defence. Soon after, proline accumulation and the *P5CS2* gene from arabidopsis were shown to be activated by plant-pathogen incompatible interactions and to result in the establishment of HR (Fabro et al. 2004), suggesting that not only proline catabolism but also its anabolism participates in plant defence to pathogens. Likewise, ProDH was shown to be induced in arabidopsis plants exposed to *Pseudomonas syringae*, in a salicylic acid (SA) dependent manner, and its inductions was shown to be important for the establishment of hypersensitive response (Cecchini et al. 2011). Similar observations were found in *Nicotiana benthamiana*, where *P. syringae* infection was observed to be enhanced by the absence of proteins involved in proline metabolism such as ProDH1, ProDH2 and OAT (Senthil-Kumar and Mysore 2012). In wheat, the transcription factor Pathogen-Induced ERF1 mediates host responses to the necrotrophic pathogen *Rhizoctonia cerealis* and was associated with proline accumulation (Zhu et al. 2014). Moreover, the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, was observed to increase the expression of proline biosynthetic genes (Zhu et al. 2014).

Because proline accumulation can be caused by the interaction of plants with non-pathogenic organisms, some authors attributed the higher stress tolerance of certain plants observed in the presence of non-pathogenic organism to the premature induction of proline accumulation. For instance, Ahmad et al. (2015) observed that the addition of *Trichoderma harzianum* to Indian mustard promoted proline accumulation in control conditions and further enhanced proline accumulation under saline stress conditions, which was explained by the authors as a mechanism by which *T. harzianum* contributes to stress tolerance. This would fit with the priming concept, in this case, exerted by *T. harzianum*. However, not yet understanding the mechanism by which proline contributes to osmotic stress tolerance, it is yet difficult to consider this as the primary reason why *T. harzianum* contributes to stress tolerance in Indian mustard. In Sect. 12.7, the potential roles of proline and its metabolism under biotic stress are discussed.

12.6 Potential Functions of GABA in Plant Response to Abiotic and Biotic Stress

GABA is recognized as a multifunctional molecule that helps plants against a plethora of stresses of both biotic and abiotic nature (Bown and Shelp 2016; Seifikalhor et al. 2019). A vast literature body positively correlates GABA to resistance against herbivory, pathogens, drought, heat, cold, salinity, high heavy metals concentrations and hypoxia (Seifikalhor et al. (2019) and references therein). Regarding abiotic stresses, evidence points to a prominent function of GABA as an osmoprotectant, contributing to maintaining cellular turgor under conditions of decreased water contents such as following salt and heat stress (Renault et al. 2011; Nayyar et al. 2014). As for proline, it is not completely understood why GABA would be a better osmolyte to accumulate than glutamate or any other amino acid. Besides helping to maintain the osmotic balance, GABA accumulation under abiotic stress was frequently associated with increased activity of the cellular antioxidant machinery, including both detoxifying enzymes, such as superoxide dismutase and catalase, and non-enzymatic scavenging systems, such as the ascorbate/glutathione cycle (Nayyar et al. 2014; Mahmud et al. 2017). It was also suggested that exogenous GABA application can boost the activities of antioxidant enzymes during the heat and heavy metal stress (Song et al. 2010; Nayyar et al. 2014), pointing to an important role for GABA in the regulation of the oxidative stress. In maize plants subjected to Cd stress, exogenous GABA treatment contributed to regulate Cd uptake, the production of ROS and PA metabolism, resulting in a greater tolerance (Seifikalhor et al. 2020). However, how GABA regulates antioxidant responses remains to be elucidated. In this respect, several authors proposed that GABA coordinates stress responses by acting as a signalling molecule in concert with phytohormones such as ethylene and abscisic acid (discussed further in Sect. 12.8) (Bown and Shelp 2016; Hijaz et al. 2018; Tarkowski et al. 2019). The suggested control of GABA over the osmotic and ROS balances can also explain the protective effects reported for GABA on the photosynthetic apparatus, a phenomenon reported during several abiotic stresses (Li et al. 2016; Kalhor et al. 2018; Seifikalhor et al. 2020).

In the case of biotic stress, GABA's capacity to modulate ROS levels and fuel the TCA cycle were linked to increased viability of host cells challenged by necrotrophs (Brauc et al. 2011; Seifi et al. 2013), together with the possibility of being involved in defence signalling pathways (McCraw et al. 2016; Hijaz et al. 2018). GABA can either supply N and C to sustain the synthesis of defence-related structures and compounds, or restrict the spread of ROS generated following HR induction in the infected area to healthy cells (Seifi et al. 2013; Tarkowski et al. 2019). In agreement with a critical role for GABA in regulating HR, studies on the bacterial hemibiotrophic pathogen *P. syringae* showed that increased GABA levels are associated with a correct HR elicitation (Kim et al. 2013; O'Leary et al. 2016).

GABA was also shown to negatively regulate quorum-sensing by binding to the bacterial protein ATU4243 secreted by *Agrobacterium tumefaciens*, inducing degradation of the quorum sensing signal and ultimately leading to decreased virulence of the pathogen (Chevrot et al. 2006; Planamente et al. 2012). Importantly, GABA has important functions also in interactions with beneficial pathogens. Root colonization by the bacterium *Pseudomonas putida* was shown to be dependent on the presence of GABA in the soil from root exudates, where it is thought to be used by bacteria as chemotactic stimulus (Reyes-Darias et al. 2015). Finally, GABA's role as an inhibitory neurotransmitter in vertebrates seems to be exploited by plants in response to herbivory (Shelp et al. 2006). Due to GABA's toxic effects on the neuromuscular junctions, its accumulation in attacked tissues leads to reduced viability and paralysis of insects and nematodes (Ramputh and Bown 1996; McLean et al. 2003).

12.7 Potential Functional Implications of Proline in Plants Under Stress

Proline over-accumulation in bacteria was observed to improve growth rate under saline stress conditions and therefore proline was proposed to contribute to osmotolerance (Csonka 1981) Likewise, in plants, both the removal of the feedback inhibition of P5CS and the expression of a transgenic P5CS resulted in higher accumulation of proline and better tolerance to water and saline stress (Kishor et al. 1995; Hong et al. 2000). Therefore, most research has attempted to correlate lines accumulating higher levels of proline with stress tolerance, but these attempts in many cases failed. Moreover, the mechanism by which proline contribute to such stress tolerance has not been described in detail at the molecular level, resulting in more controversy regarding the role of proline in osmotolerance. Two different mechanisms are believed to contribute to osmotolerance improvement, one is the osmotic adjustment and the other one is the osmoprotection of biomolecules. Regarding the osmotic adjustment, recently, Forlani et al. (2019b) estimated by theoretical calculations that, in young leaves, the levels of proline accumulated under osmotic stress are too low to contribute substantially to osmotic adjustment. On the other hand, the osmoprotection refers to the capacity of proline to surround proteins, protecting them from denaturing agents and preserving their folding. Less is known about this osmoprotective role of proline. However, under extreme conditions, such as the exposure of proteins to hydroxyl radicals, it was shown that proline can contribute to the stability of proteins, evidenced by low rates of inhibition of the enzymatic activities by hydroxyl radicals (Smirnoff and Cumbes 1989). This finding contributed to the idea that proline could act as an antioxidant by scavenging different reactive oxygen species and reducing cellular damage, including singlet oxygen (Alia and Matysik 2001; Matysik et al. 2002). However, further research demonstrated that proline is not able to protect against most ROS, including singlet oxygen (Signorelli et al. 2013a, 2016), the hydroxyl radical being an exception because they are highly reactive molecules able to react with any organic molecule, but not because proline play an antioxidant role (Signorelli 2016).

Proline was also hypothesized to function as an energy and nitrogen reservoir (Yoshiba et al. 1997), but research in this regard is needed. Under normal conditions, proline levels are much higher in young leaves than in old leaves (Masclaux et al. 2000), suggesting that proline is produced or transported to sink tissues. However, this would not explain why proline is accumulated under stress conditions. Moreover, proline accumulation and the genes regulating proline content follow diurnal cycles; in a way that proline is accumulated during the day and degraded during the night reaching control values even under stress conditions (Sanada et al. 1995; Hayashi et al. 2000). Therefore, it is unlikely that proline accumulates as a nitrogen source during stress if degraded at nights. However, proline may be accumulated as a source of energy during the day when reducing power is abundant and consumed during the night when photosynthesis cannot provide energy. This idea is linked to the hypothesis that proline is accumulated to act as a redox buffer, in which proline accumulation was suggested to promote pentose phosphate pathway during stress and proline catabolism respiration during stress relief (Hare and Cress 1997). The circadian rhythm in proline accumulations suggests that this is more likely to be relevant in a day/night basis rather than stress/non-stress basis. Recently, proline was shown to enhance leaf respiratory rates at nights (O'Leary et al. 2020), which can be seen as a mechanism to speed up the degradation of proline accumulated during the day, further confirming that either the energetic/redox role of proline seems to be more relevant in a day/night basis rather than stress/non-stress basis. Supporting the idea of an energetic/redox role, transcriptional data of the non-proline accumulating mutants, *p5cs1–4*, showed that these mutant have a differential redox metabolism in both normal conditions and low water potential (Shinde et al. 2016). This mutant also expressed more transcripts related to the mitochondrial respiratory chain (Shinde et al. 2016), suggesting that the lower catabolism of proline is reflected in lower respiration rate and an attempt of the plant to compensate for this.

The NADP regeneration by proline anabolism could not only contribute to the pentoses pathway but also serve as a final acceptor of the photosynthetic electron transport chain (Signorelli 2016). Moreover, the accumulated proline could have positive effect on photosynthetic activity by a still unknown mechanism. This is based on the fact that exogenously applied proline to maize plants subjected to osmotic stress enhanced PSII efficiency, promoted carbon fixation and chlorophyll metabolism and reduced chlorophyll degradation (Altuntaş et al. 2020). In addition, proline catabolism was hypothesized to contribute to plant autophagy (Signorelli et al. 2019), something that was demonstrated to be the case in animals (Zabirnyk et al. 2010; Liu and Phang 2012; Pandhare et al. 2015), but remains unexplored in plants.

Under biotic stress conditions, proline metabolism was also suggested to play a role (Forlani et al. 2019b). In particular, it was observed in different plants that some enzymes of proline metabolism such as OAT, ProDH, and P5CS are important for the establishment of HR, by promoting PCD (Fabro et al. 2004; Cecchini et al. 2011; Senthil-Kumar and Mysore 2012). Although early studies showed that P5CDH was also responsive to biotic stress (Ayliffe et al. 2002), more recent studies suggested that P5CDH is less relevant for the establishment of HR cell death and oxidative burst produce upon pathogen infection (Monteoliva et al. 2014).

Also, it was observed that proline metabolism can be modulated by effector-triggered immunity (Senthil-Kumar and Mysore 2012). Moreover, the regulation of proline metabolism-related enzymes under abiotic stress was shown to be affected by the simultaneous presence of biotic stress. For instance, the inoculation of arabidopsis plants with *P. putida*, can attenuate the initial upregulation of the proline biosynthetic proteins P5CS and P5CR, but resulted in a higher activity at later stages of the stress (Ghosh et al. 2018).

Another way by which proline accumulation could promote biotic stress tolerance is contributing to the biosynthesis of proline-rich proteins (PRP). Some of these proteins were shown to have a positive effect under diverse stress conditions (Kishor et al. 2015). For instance, the glycine-rich proline-rich-protein (GRPRP) of *Sorghym bicolor* plants was observed to have an antimicrobial effect and contribute to *P. syringae* tolerance (Halder et al. 2019). It is clear, that the effect of proline accumulation during biotic stress is less studied than during abiotic stress, and more evidence is necessary to understand the proline accumulation-biotic stress tolerance relationship.

Understanding in detail the functions of proline accumulation under stress is important to select cultivars having an enhanced stress response which exploits altered proline levels. Meanwhile, proline accumulation will not be incorporated as a tolerance marker in the breeding programs because, in general, the attempts to correlate levels of proline with stress tolerance have failed (Forlani et al. 2019a), as it is likely that those plants accumulating more proline are suffering from greater stress.

12.8 Potential Links Between GABA and Proline Metabolism and Hormone Signalling

Proline metabolism can be regulated via several different signal transduction pathways depending on environmental circumstance. For example, a Ca^{2+} and phospholipid based signalling mechanism is involved in proline metabolism during salt stress but not non-ionic hyperosmotic stress in arabidopsis (Parre et al. 2007). Apoplastic ROS production, as a secondary messenger, also appears to be upstream of proline accumulation, but more so in response to salt stress than in response to non-ionic hyperosmotic stress (Ben Rejeb et al. 2015). In any case, much of the discovered regulation of proline metabolism occurs at the transcriptional level and converges upon on a common mechanism of controlling P5CS and ProDH expression. There are several known cis-regulatory elements within P5CS genes, including ABA-responsive elements, which help explain why proline accumulation is a conserved response observed in a broad range of conditions in plants (Fichman et al. 2015; Aleksza et al. 2017; Zarattini and Forlani 2017). Similarly, the two

Arabidopsis ProDH genes contain differing cis regulatory elements, including for bzip transcription factors, which place them downstream of multiple signal transduction pathways (Weltmeier et al. 2006; Hanson et al. 2008). It should therefore not be surprising that there are many links between proline metabolism and hormone signalling under stress, as previously reviewed (Hare et al. 1999; Iqbal et al. 2014).

As mentioned before, the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, was shown to increase the expression of proline biosynthetic genes (Zhu et al. 2014), suggesting a potential for role of ethylene in regulating proline metabolism. In fact, in rice, the Ethylene Response Factor 71 was shown to modulate the expression of proline biosynthetic genes (*P5CS1* and *P5CS2*) and to correlate positively with drought and saline stress tolerance (Li et al. 2018b). Even more clear is the link between proline metabolism and SA or ABA. For example, there are clear links between SA hormone signalling and proline metabolism that has been observed under several stresses. Prolonged application of SA increases the abundance of proline in lentil shoots and cabbage leaves under salt stress, concomitant with an increase in P5CS activity and a decrease in ProDH activity (Misra and Saxena 2009; La et al. 2019). Likewise, the application of SA further increased the expression of *P5CS* genes and proline accumulation under drought condition in wheat (Maghsoudi et al. 2018). By contrast, direct SA infiltration leads to rapidly increased ProDH1 expression in arabidopsis leaves (Cecchini et al. 2011). During avirulent bacterial infection in arabidopsis, the increased proline content and P5CS2 and ProDH1 expression are dependent upon SA (Fabro et al. 2004; Cecchini et al. 2011). Interestingly, the exogenous application of proline to arabidopsis leaves leads to ROS burst and subsequently to the upregulation of SA synthesis indicating that the proline-SA relationship may be self-reinforcing (Fig. 12.3) (Chen et al. 2011).

ABA is another hormone widely influencing proline metabolism. In Arabidopsis and rice ABA treatment leads to increased proline accumulation and in some cases increased *P5CS* expression (Savoure et al. 1997; Strizhov et al. 1997; Ábrahám et al. 2003; Sharma and Verslues 2010). Similarly, the increase in proline upon phosphate starvation is also ABA dependent (Aleksza et al. 2017). In Arabidopsis, *ProDH1 and ProDH2* expression can also be dependent on ABA signalling under drought and phosphate stress (Sharma and Verslues 2010; Aleksza et al. 2017). Importantly, a wild variety of barley was shown to accumulate higher proline levels under drought compared to a cultivated variety because of a single nucleotide polymorphism in the *P5CS1* promoter which interrupted an ABA-responsive element, making the cultivated barley unresponsive to ABA (Muzammil et al. 2018). Therefore, the genetic variation that exists in proline metabolism response to stress that can be potentially be exploited. However, the overall regulation of proline metabolism remains seemingly complex and new mechanisms continue to be discovered.

Compared with proline, the accumulation of GABA is more clearly regulated at the post-translational level because, as discussed above, GAD activity is dependent on Ca^+/calmodulin activation (Snedden et al. 1995). However, there is increasing evidence that GABA interacts with ethylene signalling because GABA induces ethylene biosynthesis (Kathiresan et al. 1997, 1998; Shi et al. 2010). Furthermore, GABA accumulates during plant defence responses in a manner which is dependent upon ethylene biosynthesis (O'Leary et al. 2016; Tarkowski et al. 2019). It is becoming more clear that GABA itself is a signalling molecule and that many of GABA's transcriptional effects are ethylene dependent (Ramesh et al. 2017; Podlešáková et al. 2019). Interestingly, a newly discovered GABA receptor (an Al^{3+}-activated malate transporter involved in stress response) is negatively regulated by both ethylene and GABA, demonstrating at least one potential mechanism of interaction between ethylene and GABA (Tian et al. 2014; Ramesh et al. 2015; Gilliham and Tyerman 2016). Recent findings showed that GABA can repress adventitious root development in poplar and exogenous GABA was shown to accelerate the decrease of ethylene concentrations during adventitious root development as well as increase the levels of auxins, ABA and, to a lesser extent, gibberellic acids (Xie et al. 2020). Likewise, GABA was recently shown to induce auxin levels in the shoot apex of cucumber plants and polar auxin transport (PAT) through the increase of PIN1 expression (Guo et al. 2020). Given the importance of auxins in regulating several developmental transitions and stress response, this link between GABA and auxins seems worth exploring. Links between GABA and other hormones have also been observed but require further clarification (Ramesh et al. 2017; Podlešáková et al. 2019).

12.9 Upcoming Challenges for the Understanding of Proline and GABA Contributions to Stress Tolerance in Plants

This chapter has analyzed the proven and putative roles of proline and GABA metabolism and accumulation in stress conditions and has put in doubt other functions that seem to be disputed based on the current experimental evidence. Besides these ambiguities, it is clear that both molecules are involved in the abiotic and biotic stress responses. Continued evaluation of the role and relevance of GABA and proline, and closely related metabolites, will bring new information into the picture. For instance, it would be highly beneficial to identify potential receptors of either GABA- or proline-metabolism intermediates, for instance P5C, because these molecules tend to be at much lower concentrations making them potentially better signaling molecules. It would also be important to understand how the different organs and their developmental stages affect proline and GABA concentrations and how this relates to their response under stress. For instance, ripe pepper fruits (red) were showed to have much higher levels of proline than green pepper fruits (González-Gordo et al. 2019), suggesting that green and red peppers may accumulate different levels of these metabolites in response to environmental stresses. In terms of biotic stress, it is evident that the research related to proline is lagging behind that of GABA, thus, it is likely that this area of study will provide substantial new information in the near future. In terms of abiotic stress, despite the different analysis from different plant models and experimental approaches (biochemical, genetical and physiological) having contributed to gaining considerable knowledge, there is not consensus about the main role of these molecules in abiotic stress responses. Given that the synthesis and degradation pathways are highly conserved in response to stress, and so their complex transcriptional regulation, it seems plausible that these molecules are linked to multiple functions at the same time. Therefore, it would be necessary to apply statistical tools that allow the integration and comparison of multiple variables at the same time, being each of these variables related to markers of the different roles to be assessed. In addition, more experimental evidence wherein proline and GABA accumulation and metabolism are associated with plant survival to a stress episode are necessary. In the near future, through the application of computational models, omics technologies, and editing genome techniques, abundant and varied information about the functions of these molecules in the tolerance mechanism to abiotic and biotic stress should be consolidated. In this way, since proline and GABA have distinct and particular roles in plants and these are species-specific, a profuse characterization of the roles these molecules play during specific stress conditions in certain plant species could contribute to identifying helpful indicators in order to classify genotypes with contrasting stress responses. Thus, these molecules can be used as functional markers of stress tolerance in order to select tolerant genotypes in breeding programs. Finally, a deeper understanding of the capacity of phytohormones to control proline and GABA metabolism would researchers to strategically manipulate a phytohormone's metabolism to ultimately enhance the control of proline and GABA metabolism.

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