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REVIEW

γ-Aminobutyric acid (GABA) signalling in plants

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Abstract The role of γ-aminobutyric acid (GABA) as a signal in animals has been documented for over 60 years. In contrast, evidence that GABA is a signal in plants has only emerged in the last 15 years, and it was not until last year that a mechanism by which this could occur was identified—a plant 'GABA receptor' that inhibits anion passage through the aluminium-activated malate transporter family of proteins (ALMTs). ALMTs are multigenic, expressed in different organs and present on different membranes. We propose GABA regulation of ALMT activity could function as a signal that modulates plant growth, development, and stress response. In this review, we compare and contrast the plant 'GABA receptor' with mammalian $GABA_A$ receptors in terms of their molecular identity, predicted topology, mode of action, and signalling roles. We also explore the implications of the discovery that GABA modulates anion flux in plants, its role in signal transduction for the regulation of plant physiology, and predict the possibility that there are other GABA interaction sites in the N termini of ALMT proteins through in silico evolutionary coupling analysis; we also explore the potential interactions between GABA and other signalling molecules.

Keywords γ-Aminobutyric acid · Aluminium-activated malate transporters · $GABA_A$ receptors · Signalling · GABA metabolism · Carbon–nitrogen balance · Stress response · Topology · Pharmacology

Abbreviations

3-MPA	3-Mercaptopropionic acid
ALMT	Aluminium (Al^{3+})-activated malate transporter
C/Cys	Cysteine
EC_{50}	Half-maximal response
F/Phe	Phenylalanine
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase
GABP	GABA permease
GAD	Glutamate decarboxylase
GAT	GABA transporter
GDH	Glutamate dehydrogenase
E/Glu	Glutamic acid
I/Ile	Isoleucine
SSA	Succinic semialdehyde
SSADH	Succinic semialdehyde dehydrogenase
T/Thr	Threonine
D/Asp	Aspartic acid
V/Val	Valine
Y/Tyr	Tyrosine
Q/Gln	Glutamine
L/Leu	Leucine
R/Arg	Arginine
TMDs	Transmembrane domains
K/Lys	Lysine
S/Ser	Serine
G/Gly	Glycine

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Introduction

The non-proteinogenic amino-acid γ-aminobutyric acid (GABA) was first isolated in 1949 from potato tubers [1], prior to its discovery in animal brain extracts [2]. Soon after, in the 1950s and 1960s, evidence was gathered that suggested GABA might act as an inhibitory neurotransmitter in animals; GABA was found to suppress impulses generated by crayfish stretch receptor neurons [3, 4]. Yet, it was not until Bloom and Iversen [5] that GABA was localised to mammalian nerve terminals, and it took a further 10 years until the mechanism by which GABA acts as an inhibitory neurotransmitter was identified—via its activation of $GABA_A$ (ionotropic) and $GABA_B$ (metabotropic) receptors [6]. In mammals, GABA counteracts the action of excitatory neurotransmitters in the mature brain [5], through the activation of a chloride (Cl^-) conductance that passes through $GABA_A$ receptors into mature neurons leading to membrane hyperpolarisation [7]. This prevents the neurons from firing and thus has a calming effect [8]. Its action has been mainly described in the nervous system, where GABA receptors regulate brain function and development [9, 10], although GABAergic receptors have also been described as functioning in other tissues beyond neuronal cells, such as human organs [11, 12]. This has been extensively reviewed [10, 13]. GABA as a signalling molecule in animals has been studied over six decades, whereas in plants, it is mostly defined as a carbon–nitrogen metabolite [14–16]. This said, evidence has been mounting, since the 1990s that GABA may act as a signal in plants, including: (1) GABA concentration in plant tissue is variable (0.03 $\sim$ 6 μmol g^{-1} fresh weight) and prone to large and rapid increases (<1000-fold) following exposure to a multitude of biotic and abiotic stresses [17, 18]; (2) GABA concentration gradients can be found in plant tissues [19, 20]; (3) GABA metabolism is compartmentalised intra- and inter-cellularly [21]; (4) GABA and GABA receptor agonists and antagonists alter plant growth [22]; (5) GABA binding sites have been detected on plant cell membranes [20, 23], and recently, (6) the identification of GABA-regulated ion channels in plants that also have their activity regulated by drugs known to affect GABA receptors in animals [18].

A number of reviews have been published in the past two decades, which have summarised plant GABA metabolism and its contribution to plant growth, development, and stress adaptation [16, 17, 22, 24–26]. However, the discovery that a family of plant anion channels, the aluminium (Al^{3+})-activated Malate Transporters (ALMTs), are regulated by GABA, and this regulation can modulate tissue growth [18] warrants a re-examination of the roles of GABA in plants. In particular, this regulation has been proposed to transduce GABA metabolism into membrane signalling via an alteration of anion flux across cell membranes [27]; as such, this discovery opens novel research avenues for plant and animal biology [28].

Despite being an anion channel—such as animal $GABA_A$ receptors—ALMTs were observed to share little sequence homology with their proposed animal counterparts, except in a 12 amino stretch that has some similarity to one important motif for GABA binding in rat $GABA_A$ receptors [18]. Whilst GABA activates $GABA_A$ channel activity in mammals [13], GABA inhibits ALMT activity in plants [18]. However, as the equilibrium potential for chloride is generally positive in plants and negative in mature animal neurons, this GABA-regulated anion flux (respectively, inhibition in plants and activation in animals) leads to a relative hyperpolarised state in the cells of both kingdoms [18, 28]. Changes in membrane potential are a key cellular signal, so the finding that GABA alters this in plants, and that this is a prerequisite for changes in tissue growth [18], suggests that GABA can act as a signal in plants. The fact that GABA can be present in large concentrations and occurs in every part of the plant examined has been used as an argument against GABA being a signal in plants [14]. For instance, it can be the main amino acid found in tomato fruit ($\sim$11.5–20 mM) [29], and during stress, it can often exceed the levels of all other amino acids [22]. The same argument was used against GABA being a signal in animals in the 1950s and 1960s, until the receptor proteins were identified and local gradients of GABA discovered [30, 31], we now have similar evidence in plants (Table 1).

In this review, we will provide an update on GABA-regulated ion channels in plants and explore their possible linkage with GABA-mediated physiological processes to provide an insight into the putative roles of GABA signalling in plant biology. In the first part of this review, we will compare and contrast ALMTs with animal $GABA_A$ receptors in terms of their molecular identity, predicted topology, mode of action, and signalling roles. The aim of this section is to ascertain what the commonalities and differences are between GABA signal transduction in animals and plants. In the second part of this review, we focus on the unique effects that GABA has on plants and we explore the implications of the discovery that GABA regulates ALMT activity for transducing signals for the regulation of physiological processes, and the potential interactions between GABA and other signalling molecules.

Plant ALMTs vs. animal $GABA_A$ receptors

ALMTs are likely to be involved in signalling

ALMT proteins encode voltage-dependent anion channels [32, 33] and in at least one case a Rapid or Quick activating

Table 1 GABA distribution in different plant organs and species

Species	Organs	GABA concentration	References
Arabidopsis thaliana	Root	~0.40–0.1 μmol g^{-1} FW/ ~8 μmol g^{-1} DW	[19, 161, 162, 171, 179, 188, 270–272]
	Shoot	~0.03–1 μmol g^{-1} FW/ <1 μmol g^{-1} DW	
	Flowers	~0.2 μmol.g^{-1} FW	
Nicotiana tabacum	Pistil	~0.6–4 μmol g^{-1} FW	[258]
	Shoot	~0.2–1 μmol g^{-1} FW	[207, 270]
	Root	<0.2 μmol g^{-1} FW	
	Seedling	~25 μmol g^{-1} FW	[273]
Nicotiana sylvestris	Leaf	~10 μmol g^{-1} FW	
Brassica napus	Root	~0.5 μmol g^{-1} FW/ ~3.6 μmol g^{-1} DW	[164]
	Leaf	~1.30 μmol g^{-1} FW/ ~ 1.1 μmol g^{-1} DW	[184]
Oryza sativa	Calli	~0.2–0.3 nmoles g^{-1} FW	[257, 274, 275]
	Root	~0.5–1 μmol g^{-1} FW	
	Shoot	<0.5–1 μmol g^{-1} DW	[187, 276]
	Kernel	~0.01–0.12 μmol g^{-1} FW	[277]
	Embryo	<5 μmol g^{-1} FW	[278]
Glycine max	Xylem	~100–160 μM	[196, 279, 280]
	Leaf	~0.05–0.4 μmol g^{-1} FW	
	Root	~0.1 μmol g^{-1} FW	
	Nodule	~1.5 μmol g^{-1} FW	
	Seedling	<1 μmol g^{-1} FW	[180]
	Cotyledon	~25 μmol g^{-1} DW	[185]
	Embryo	~15 μmol g^{-1} DW	
Medicago sativa	Root	~0.4 μmol g^{-1} FW	[189]
	Nodule	~2.4 μmol g^{-1} FW	[248]
	Phloem	~1.4 nmol g^{-1} FW	
Solanum lycopersicum	Fruit	~0.5–40 μmol g^{-1} FW	[281–283]
	Leaf	~3–5 μmol g^{-1} FW	[8, 29, 266]
Triticum aestivum	Root	~2–4 μmol g^{-1} FW	[18, 284]
	Seedling	~0.02 μmol g^{-1} FW	[178]
Hordeum vulgare	Seedling	~0.02 μmol g^{-1} FW	[178]
Eriobotrya japonica	Fruit	~0.15–0.35 μmol g^{-1} FW	[234]
Cucumis melo	Root	~0.25 μmol g^{-1} FW	[285]
Vicia faba	Bean	<10 μmol g^{-1} DW	[286]
Vitis vinifera	Berry	~1.4 μmol g^{-1} FW	[287]
Comellia sinesis	Leaf	~15 μmol g^{-1} DW	[288]
Phaseolus vulgaris	Leaf	~4.4-9 μmol g^{-1} DW	[195]
Pisum sativum	Nodule	<1.5 μmol g^{-1} FW	[289]
Caragana intermedia	Root	<0.05 μmol g^{-1} FW	[230]

GABA has been found in all organs in plants, including embryo, cotyledon, roots, shoot, flowers, fruit, nodule, xylem, and phloem

A GABA gradient exists increasing from top to bottom in flower pistils

FW fresh weight, *DW* dry weight

Anion Channel (R/QUAC-type) [34]. As in animals, anion channels have been demonstrated to be important signalling proteins in plants. Processes that depend on the function of R-type anion channels include blue light and auxin inhibition of hypocotyl growth [35, 36] and ROS production in response to bacterial pathogens [37]. When anion channels open, anions are released from the cell tending to depolarise the membrane voltage from its normally very negative resting level [38]. ALMTs are activated by some anions when placed on the efflux side of the channel protein [18, 33]. Such transactivation is observed in vivo for the R-type anion channels of stomatal

guard cells, vacuoles, and hypocotyls [37]. Transactivation may serve to keep anion efflux occurring through the channel in the face of a decreasing gradient. When potassium (K^+) channels open in response to depolarisation, caused by activation of anion channels, the combined effect is loss of osmoticum and reduced turgor pressure. Stomatal pore closure, i.e., loss of guard cell turgor, relies on this process and involves R-type and other anion channels [39]. A sensing and signalling role for R-type channels has been suggested [34]. Figure 1 summarises the factors that regulate the R-type channels and ALMT anion channels.

GABA is a key regulator of ion channels in plants and animals

In mammals, GABA can open channels via the activation of either $GABA_A$ or $GABA_B$ receptors [40]. $GABA_A$ receptors are Cl^- channels [6], whilst $GABA_B$ receptors are G-protein coupled receptors that regulate cation transport (e.g., K^+ and Ca^{2+}) [41]. The ionotropic $GABA_A$ receptor family also includes $GABA_A$-rho receptors that are only composed of rho (ρ) subunits which form distinct ligand-gated Cl^- channels; these were previously designated as $GABA_C$ receptors [31]. GABA is also involved in proliferation, differentiation, and migration of different kinds of cells in animals, including cancer cells [13]. In contrast to its action in mature cells, GABA can depolarise immature neurons due to different equilibrium potentials for Cl^-, trigger sodium action potentials, increase internal calcium (Ca^{2+}), reduce the voltage-dependent magnesium block of NMDA channels, and interfere with ionotropic glutamatergic transmission [42, 43].

For plants, an early candidate, touted as a receptor for GABA signalling, was the plant glutamate receptor-like proteins (GLRs), which have high sequence similarity to animal ionotropic glutamate receptors (iGluRs) [44]. These

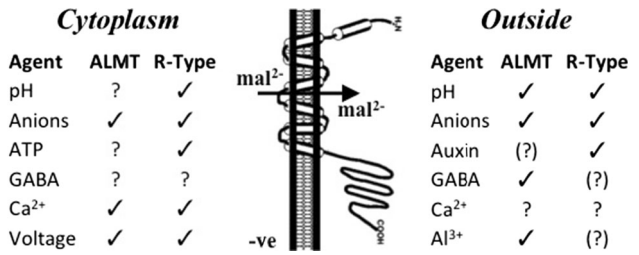

Fig. 1 Summary of known and possible factors that control the activity of ALMT anion channels and R-type anion channels. A *tick* indicates similar responses in gating and/or voltage dependence, *question mark* indicates not known, and *question mark* with *brackets* indicates possibility based upon indirect evidence. Note that ALMTs do not necessarily respond to all these factors, similarly for R-type channels

possess a regulatory domain with structural homology to the animal $GABA_B$ receptors [45–47]. They are involved in glycine and serine signalling [48, 49], are thought to play a role in Ca^{2+} utilisation, and stimulate transient changes in cytoplasmic Ca^{2+} concentrations, as they behave as ligand-gated Ca^{2+} channels [48–52]. Thus, in plant cells, if GABA interacts with GLRs, it should cause transient elevations in cytosolic Ca^{2+} [48, 52]; however, in *Arabidopsis thaliana* seedlings, GABA (1 mM) did not induce changes in Ca^{2+} levels [53]. Notwithstanding this negative result, it is possible for membrane potential transients elicited via GABA inhibition of ALMTs to indirectly result in cytoplasmic Ca^{2+} transients via hyperpolarisation-activated Ca^{2+} channels [54, 55].

In plants, GABA appears to negatively regulate ALMT-mediated anion flux [18]. There are multiple ALMTs in all plants, and all those tested by Ramesh et al. [18], from wheat, barley, grapevine, *Arabidopsis,* and rice were sensitive to low micromolar concentrations of GABA. An ALMT from *Arabidopsis* carries a R-type anion conductance across the plasma membrane, whereas other ALMTs are localised to the vacuolar membrane and are involved in the passage of malate and chloride across the tonoplast [56, 57]. Both types of conductance are ubiquitous in plant cells and have been shown to be, or are implicated to have signalling roles in plants; for instance, in processes, such as pathogen responses, the control of gas exchange, pollen tube growth, and in response to drought, salt, and acidosis [58–60] and references therein [18, 61]. As a consequence, ALMT proteins appear to be clear candidates for transducing GABA and other signals in all plant cells.

GABA research in plants thus far has focused more on how its metabolic roles and its synthesis during stress can ultimately impact plant growth. GABA-regulated processes are thought to include developmental regulation, pH regulation, stress tolerance, carbon:nitrogen balance, and long-distance transport (reviewed in [14, 21, 62]). Here, we speculate that some of the physiological processes affected by GABA may involve GABA-modulated signal transduction via ALMT or possibly the activity of other as yet unconfirmed 'receptors' (see "GABA regulates plant growth and development").

Structure and topology of plant ALMTs vs. mammalian $GABA_A$

The ALMT family widely exists in land plants, but no homologs have been identified in mammals [58]. Although ALMTs and animal $GABA_A$ receptors are both anion channels, they share little similarity in protein sequence, except in a 12 amino stretch important for their regulation by GABA [18]. The $GABA_A$, nicotinic acetylcholine

(nAChr), $GABA_A$-ρ, glycine, and 5-HT_3 receptors are members of cysteine (Cys) loop ligand-gated ion channel superfamily. The structure of $GABA_A$ receptors in mammals has been well characterised [63–65]. They are members of the pentameric ligand-gated ion channels (pLGICs), which are ubiquitous neurotransmitter receptors in animals and certain prokaryotic homologues, but are completely absent from multicellular plants and fungi [66]. The eukaryotic members share a motif composed of two Cys residues separated by 13 amino-acid (aa) residues [66], and the $GABA_A$ receptors from different animal species are highly conserved. The mammalian $GABA_A$ receptor heteromer is composed of three subunits α, β, and γ, which are associated in a defined ratio to form a functional receptor [67, 68]. The ligand-binding sites are localised at the β (+) and α (−) interfaces [69, 70], with both α and β subunits being essential for GABA binding, whilst the subunit composition within the receptors is dependent on the brain regions or on species [71]. For instance, 19 different subunit compositions have been identified in humans that determine the differential GABA-binding affinities of $GABA_A$ receptors and these properties suggest that they can translate diverse GABA signals shaped by development into a functional response [72]. The subunit heterogeneity of $GABA_A$ receptors determines to some extent whether it mediates phasic (shorter-lasting inhibition typically generated by the activation of $GABA_A$ receptors following action potentials in a presynaptic interneuron) or tonic (long lasting inhibition generated by GABA conductance activated by GABA in the extracellular synapses) inhibition, as reviewed in [72–76]. The five subunits of $GABA_A$ receptors form a central pore that remains closed under normal conditions but opens following a conformational change induced by GABA binding [77, 78]. Typically, a mature subunit is ~450 aa in length and has a hydrophilic extracellular N-terminal domain that contains the Cys loop which is the site of action for various drugs, followed by four hydrophobic transmembrane domains (M1 to M4) and a short C-terminal domain. A role for two extracellular Cys residues in agonist binding to the receptor had been suggested [66], but the subsequent mutational studies in $GABA_A$, nAChR, and glycine receptors suggest otherwise [79–81]. From the solution of the crystal structure of $GABA_AR$, the human β3 homopentamer, details of the ligand-binding pocket, and key residues in the interaction with agonist were identified and these support previous studies identifying key residues in ligand binding for nAChR [82]. The transmembrane domain M2 lines the channel pore and between M3 and M4 is a long intracellular loop that is involved in modulation of the receptor by phosphorylation, protein–protein interactions, and post-translational modifications [83, 84] (Fig. 2a, b). A number of proteins that are involved in receptor trafficking and anchoring of receptors to the cytoskeleton and post-synaptic membrane interact with the intracellular loop [85, 86]. It is clear that separate regions on the extracellular domains of the N terminus form the binding pocket, including regions on adjacent subunits. The $GABA_AR$ β3 homopentamer comprises regions in a principal face (loops A–C) and a complementary face on an adjacent subunit comprising regions of loops D (Tyr^{62}–Gln^{64}) and E (Leu^{125}–Arg^{129}). It is the region of loop D (also referred to as β2 strand) that was found to show some similarity to a 12 residue "motif" in plant ALMTs and a critical phenylalanine that when mutated to cysteine virtually abolished GABA sensitivity [18, 87].

In comparison with animal GABA receptors, the structure of ALMTs is poorly understood. It is not known whether the channels are monomeric or can form multimers consisting of homomeric or heteromeric combinations—although we are aware that this is an active area of research. The ALMT genes form a functional protein when expressed alone in *Xenopus laevis* oocytes but whether the channel is formed from multiple subunits or whether a functional GABA-binding site can occur in a monomer is not clear. The region of similarity between rat $GABA_A$ receptor and TaALMT1 is localised at the N terminus of the former and the C terminus of the latter. Several studies have predicted the putative TaALMT1 topology [58, 88], but the models differ; one suggests that TaALMT1 has six transmembrane domains (TMDs) and its N- and C terminus both face the extracellular space [88], whereas the other, based on phylogenetic analysis of ALMTs across the plant kingdom, predicts that TaALMT1 possesses eight TMDs and its N- and C-terminus are localised to the inside and the outside of cells, respectively [58] (Fig. 2c, d). The evidence from the rapid inhibition of malate efflux in *X. laevis* oocytes expressing *TaALMT1* by external GABA suggests that the interacting site is localised at the extracellular side or at least rapidly accessible to an intracellular or transmembrane site [18]. Interestingly, based on YFP-QUAC1 (rapid-type anion channels, e.g., AtALMT12) fusion studies, Mumm et al. [89] predict that both the N and C termini are located in the cytosol. In silico analysis of *Arabidopsis* ALMT9 located on the vacuolar membrane predicts six TMDs with N terminus facing the cytoplasm [90]. A predicted-soluble C-terminal domain encompasses nearly half of the protein. Patch clamp analysis of amino-acid mutations in AtALMT9 revealed that individual residue affected the function of the channel differently. The removal of positive and negative charges (Lys^{93}, Lys^{187}, Arg^{143}, Arg^{226}, and Glu^{130}) abolished its conductivity. Mutation of Arg^{200} and Arg^{215} affected channel function depending on which residue was substituted, and mutation of these residues to asparagine resulted in time-dependent inward currents comparable to

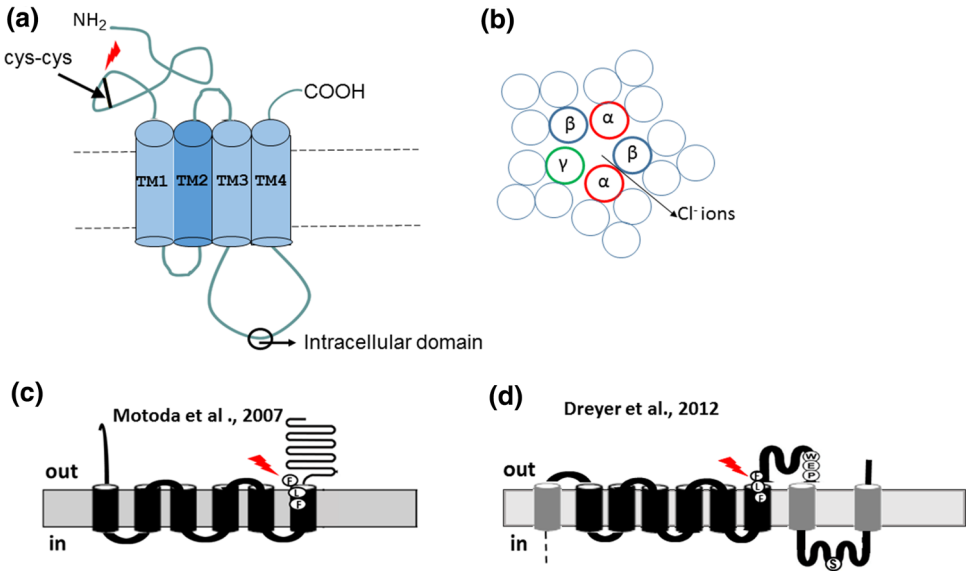

Fig. 2 **a**, **b** Schematic representation of $GABA_A$ receptor ($GABA_AR$) structure. **a** Transmembrane topology reveals that each subunit is composed of four hydrophobic TMDs (TM1-4), a large extracellular ligand-binding NH_2 region with a disulphide bond characteristic of the family of cys-loop receptors, and a short barely extruding COOH terminus. The residues important for GABA-binding reside at the N terminus and are indicated by the *red arrow*. Each subunit also contains a large intracellular domain between TMs 3 and 4 which is site of protein–protein interactions and also undergoes numerous post-translational modifications. **b** Transverse view of the subunits that form an ion channel. TM1 and three interact with neighbouring subunits, TM2 faces the lumen of the ion channel, while TM4 is anchored in the lipid membrane. **c**, **d** Schematic representation of predicted topology models of wheat (Ta) ALMT1. **c** Model proposed by Motoda et al. [88] predicts both N and C termini to be extracellular with six transmembrane domains. The residues important for GABA binding reside at the end of six TMs and are indicated by the *red arrow*. **d** Second model proposed by Dreyer et al. [58] predicts that in addition to six transmembrane domains, the large N terminus may contain another transmembrane domain (shown in *grey*). Furthermore, the large C terminus may span the membrane twice resulting in intracellular and extracellular domains (shown in *grey*). The position of the residues important for GABA binding is indicated by the *red arrow,* and the highly conserved WEP motif and phosphorylation site (S384) are also shown

wildtype AtALMT9 currents, whereas mutation to Glu (E) resulted in loss of channel function. Furthermore, the sensitivity of point mutations of AtALMT9 to open-channel blocker citrate suggested that Lys^{193} and Arg^{200}, which are located near or within TMα5 are part of the ion conduction pathway of AtALMT9 [90]. Functional analysis of site-directed mutant K193E (insensitive to citrate block) by patch clamping, and AtALMT9-GFP studies, suggested that AtALMT9 functions as a multimer composed of four subunits [90].

Amino-acid residues important for GABA binding in the $GABA_A$ receptors were identified by photoaffinity labelling of an affinity purified bovine receptor with [^{3}H] muscimol and microsequencing of a purified labelled peptide [91]. Mutational analysis has identified the key residues essential for GABA binding to the α1 subunit of rat $GABA_A$ receptors through a point mutation of Phe^{64} [92] (Fig. 3). This mutation reduced the affinities of both agonist and antagonists of rat $GABA_A$ [92]. Furthermore, it has been observed that a similar mutation in α5 subunit had the same effect suggesting that there are close functional and structural associations of α-subunits with binding sites [92]. Substituted cysteine accessibility method (SCAM) analysis of the amino acids in the proposed binding region (α_1 Tyr^{59}–Lys^{70}) mutated to Cys and expressed with wild-type β subunits in HEK293 cells confirmed that F^{64}, R^{66}, and S^{68} residues line part of the binding site and that Phe^{64} (α_1F^{64}) was very important in GABA binding [87]. Similar studies in the β2 subunit confirmed that Tyr^{97} and Leu^{99} line the GABA-binding site [87]. However, Szczot et al. have shown that rapid application of agonists to rat recombinant $\alpha_1\beta_2\gamma_2$ receptors with the α_1F^{64} mutations affected gating, abolished rapid desensitization, slowed current onset, and accelerated deactivation [93]. Further α_1F64C mutation resulted in a decrease in open-channel probability without affecting channel conductance.

Similarly, in plants, site-directed mutagenesis has been performed to probe a putative GABA binding site. In *TaALMT1* mutagenesis of Phe^{213} (F^{213}), residue appears to affect affinity of GABA action increasing EC_{50} from 3.4 μM to 1.8 mM [18] suggesting that this residue might be important for GABA binding. However, it is yet to be demonstrated that the mutation of F^{213} to C in TaALMT1 affects gating or sensitization (Table 2). The mutation of equivalent aromatic residue Y (Tyr) in *Vitis vinifera* VvALMT9 to C (Cys) increases EC_{50} from 6.0 to 380 μM.

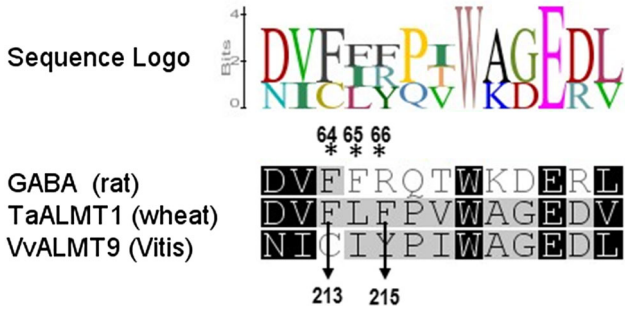

Fig. 3 Sequence alignment of rat $GABA_A$ α subunit with wheat TaALMT1. Residues important for GABA sensitivity indicated by an *asterisk* in the rat $GABA_A$ α subunit, while *arrows* point to the residues important for GABA sensitivity in TaALMT1. Alignment was performed with Geneious 9.0.4 using CLUSTAL and sequence logo was also generated using Geneious 9.0.4. The *scale bar* to the *left* of the graph shows minimum and maximum coverages for the alignment, as well as a *tick* somewhere in between for the mean coverage. The height of the logo at each site is equal to the total information at that site and the height of each *symbol* in the logo is proportional to its contribution to the information content

Nevertheless, these mutations do not completely abolish GABA sensitivity of TaALMT1 [18], and as such, there may be other regions that affect GABA sensitivity and likely binding of GABA [27]. An in silico analysis of 116 different ALMTs revealed that the putative GABA-interaction motif appeared highly conserved across a wide range of plant species [18]. A protein–protein BLAST search of *Arabidopsis* proteins using a consensus sequence generated from the $GABA_A$ and ALMT regions of similarity [18] identified the majority of ALMT members in *Arabidopsis* as well as other proteins, such as putative F-box protein, ACT-like protein tyrosine kinases-like, and an uncharacterised protein (Tables 3, 4). However, we do not know if all or any of these identified proteins are targeted to cell membranes or catalyse ion transport [94, 95]; therefore, if they do bind GABA, they may act through a novel mechanism. It is also possible that the consensus motif alone may not be sufficient to confer protein GABA-binding ability, and other important regions in ALMTs are also essential.

Although no tertiary structure for ALMTs has been resolved experimentally, there are bioinformatic techniques that can predict this and potential ligand-binding sites in a protein. One technique involves examining homologous protein sequences across a wide range of organisms, and provided there are enough sequences, it is possible to examine the co-evolution of amino-acid residues in a protein [96]. If there is evolutionary coupling between residues, it would imply that they are linked structurally and that they are located near to each other in the tertiary structure [97]. This can be then used to predict folding in the protein. This technique known as evolutionary coupling analysis has been used on several proteins to provide

Table 2 Effect of mutations on residues important for GABA binding

Name	Wild-type residue	Affinity (EC_{50} μM)	Mutation	EC50 (μM)
$GABA_A$ α1(rat)	F^{64}	594	F^{64} to C^{64}	72.8
	F^{65}	19	F^{65} to C^{65}	2.34
	R^{66}	2610	R^{66} to C^{66}	320
TaALMT1	F^{213}	3.4	F^{213} to C^{213}	1000
	F^{215}	3.4	F^{213}/F^{215} to C^{213}/C^{215}	1853

structure predictions that turn out to be very close to known structures from X-ray crystallography, including those for complex ligand-activated ion channels [97]. In the context of ALMTs, there are now thousands of homologous protein sequences in the data bases and these can be harvested to examine evolutionary coupling between residues and to provide insight into residues in TaALMT1 that may be involved in GABA binding. Submitting the TaALMT1 sequence to the Web portal EVFold provides data on the coupling between residues over evolutionary time (utilizing 3688 sequences) and identifies "hotspots" in the protein's evolution indicating important functional sites [97, 98]. Interestingly, residues in the putative GABA motif, including F^{213}, show significant evolutionary coupling (in the top 50 for the protein) with residues in the N terminus (Fig. 4). These are potential residues involved in forming a GABA-binding pocket [96]. Using these couplings and other information about likely secondary structure and transmembrane domains, the EVFold computation also predicts tertiary models of the protein of interest. The top-ranking model is shown in Fig. 4 and displays some of the evolutionarily coupled residues and their proximity to F^{213}. In this region, the model predicts that the aromatic side chain is exposed and can form a cavity in the protein, which is tempting to speculate may accommodate a GABA molecule. Two residues R^{40} and Y^{96} (among a total of seven residues) at the N terminus and start of the first TM showed a significant evolutionary coupling with F^{213} in the GABA interaction motif. This information provides the basis to test the model by site-directed mutagenesis, particularly of the residues identified as being closing coupled.

Trafficking, movement and endocytosis

The regulation of the $GABA_A$ receptor in animals depends on the number of receptors at the post-synaptic membrane either via expression, lateral movement, endocytosis, or rate of re-insertion of the receptors into the membrane. Numerous studies have been carried out to understand these processes in glycine and AMPA receptors, but

Table 3 Regions in other proteins that may have a role in GABA binding

Proteins	Equivalent sequence	Coverage (%)	Identity (%)	Accession
ALMT5	NVFLFPIWAGEDL	100	38	NP_564935.1
ALMT6	NIFIFPIWAGEDL	100	31	NP_179338.1
ALMT4	NIFILPIWAGEDL	100	31	NP_173919.1
ALMT8	IFICPVWAGEDL	93	33	NP_187774.1
Putative F-box protein	VFAPPNWFGEPL	92	42	NP_177195.1
ACT-like protein tyrosine kinase-like protein 8, STY8	DVFVVDGWSQE	84	45	NP_179361.1
ACT-like protein tyrosine kinase-like protein17, STY17	DVFVVDGWSQE	84	45	NP_195303.2
ACT-like protein tyrosine kinase-like protein 46, STY46	DVFVVDGWPYE	84	45	NP_568041.1
Uncharacterized protein	EVFGVVIWKKE	84	36	NP_193542.1

Amino-acid regions identified using BLAST search using consensus sequence "DVFXXXXWXXEXL" (coverage above 80% only listed below)

Table 4 Regions in *Arabidopsis* proteins that may have a potential role in GABA binding

Description	Equivalent sequence	Coverage (%)	Identity (%)	Accession
ALMT10	VFFCPIWAGSQL	92	58	NP_567199.2
ALMT5	NVFLFPIWAGEDL	100	54	NP_564935.1
ALMT6	NIFIFPIWAGEDL	100	46	NP_179338.1
ALMT4	NIFILPIWAGEDL	100	46	NP_173919.1
putative F-box protein	VFAPPNWFGEPL	92	58	NP_177195.1
ALMT8	IFICPVWAGEDL	92	50	NP_187774.1
ALMT9	NMFIYPIWAGEDL	100	46	NP_188473.1
ALMT14	VF–PIWSGEDL	92	58	NP_199473.1
ALMT12	VF–PIWSGEDL	92	58	NP_193531.1

Amino acids identified using BLAST search with GABA binding motif "DVFFXPTWXGEXL" (coverage above 90% only listed)

relatively little has been published in this regard about $GABA_A$ receptors [99, 100]. However, it has been shown that $GABA_A$ receptors behave in a similar manner to the glycine and AMPA receptors in that there are both mobile and immobile receptor pools that move laterally in the membrane to regulate the $GABA_A$ receptor concentrations to adjust to changing environments [101]. In plants, nothing much is known about the trafficking of ALMTs to the plasma membrane or its movement in response to various abiotic or biotic stresses.

Mammalian $GABA_A$ receptors are constitutively endocytosed and recycled back to the surface of the membrane to regulate the efficacy of the GABAergic transmission [102, 103]. Briefly $GABA_A$ receptors undergo endocytosis via clathrin coated pits [104] by binding of the β and γ subunits to the clathrin adaptor AP2 [105] and require a di-leucine motif for efficient endocytosis [105, 106]. Furthermore, the expression of the $GABA_A$ receptors might be downregulated by exposure to GABA and benzodiazepine agonists [107, 108]. Preliminary data in plants suggest that GABA-mediated inhibition of anion flux is not regulated by endocytosis [18], but more extensive research is needed to understand how GABA regulates plant processes.

Pharmacological comparison of ALMTs with GABA receptors

Numerous plant-derived and synthetic pharmacological agents have been used to characterise animal GABA receptors and their role in signalling (either as agonists or as antagonists) [109, 110]. These include muscimol, bicuculline, vigabatrin, and 3-mercaptopropionic acid (3-MPA) [111, 112]. Muscimol (as an agonist) and bicuculline (as an antagonist) are commonly applied to mammalian $GABA_A$ receptors expressed in heterologous systems to mimic and block GABA action, respectively, via their interaction with the GABA-binding site [113–115]. Effects of both drugs have also been observed on ALMTs expressed in *X. laevis* oocytes. Muscimol, like GABA, reduces TaALMT1-mediated malate efflux, but the application of bicuculline

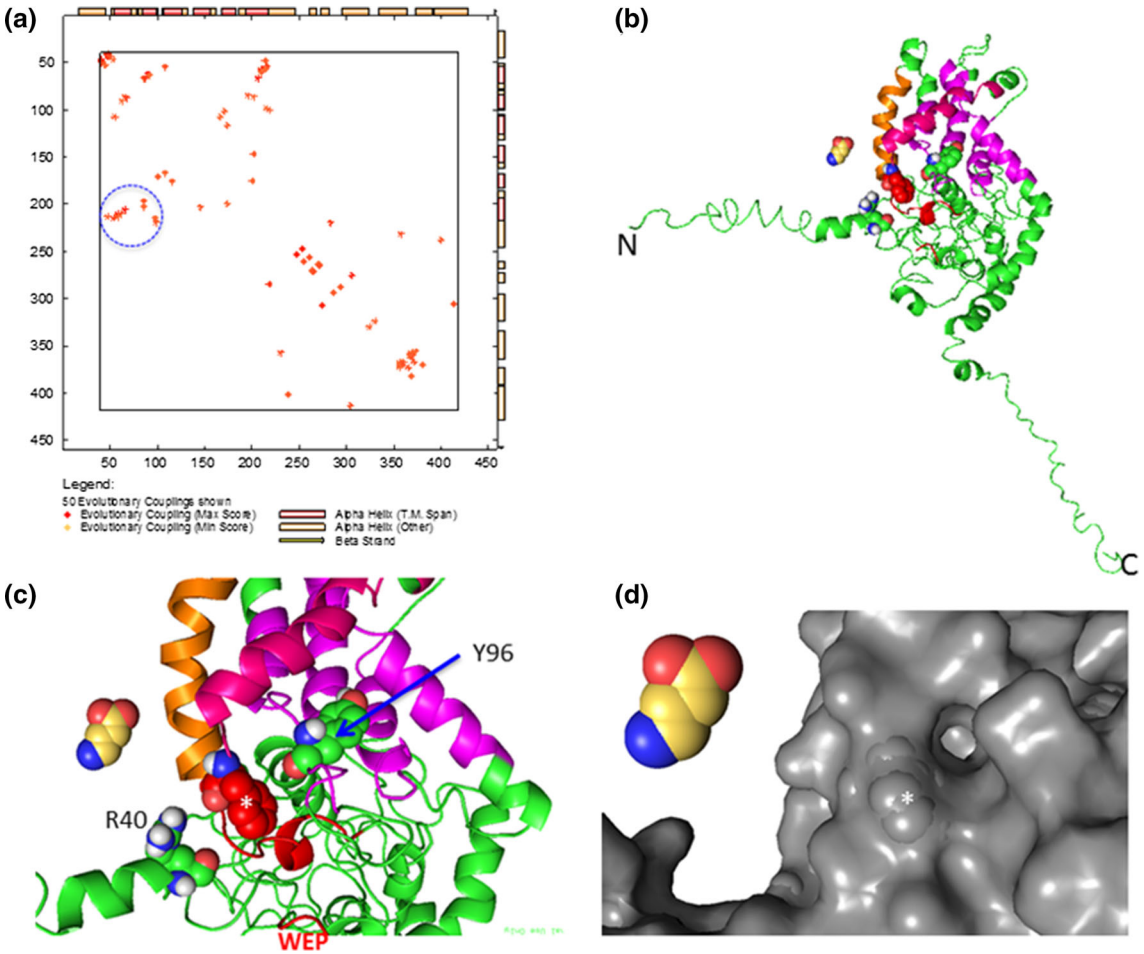

Fig. 4 One 3D model of TaALMT1 protein computed from evolutionary sequence variation using the EvFold web portal. **a** Top 50 modeled contacts computed from co-evolution of residue pairs from 3688 alignments using TaALMT1 as input with overall *E* value of 10^{-5}. The *circled* region denotes the region of amino acids that includes the putative GABA interaction motif and F213, and showing significant coupling to a short region in the N terminus and as a hotspot in the evolution of the protein. The diagram on the *top* and *right* sides of the plot denote secondary structure predictions of helices (*yellow*) and transmembrane helices (*red*). **b** Computed 3D model from EVFold illustrating six transmembrane (TM) domains (*orange*, *cyan,* and *red*) with N terminus first TM denoted orange and the sixth TM denoted *red*. The F213 is at the C terminus end of TM6. N and C termini are predicted to be on the cytoplasmic side. The GABA molecule is shown as size comparison. **c** Close-up of the GABA interaction motif showing F213 (*asterisk*) and two residues at the N terminus and start of the first TM that showed significant evolutionary coupling (R40 and Y96 among a total of six residues). The aromatic side chain of F213 forms a surface of a cavity when examining the protein surface plot (**d**). Another cavity is present between F213 and R40 on the N terminus. *Diagram* in **a** was obtained from the output files of EvCouplings and images of the 3D structure were drawn with PYMol from the downloaded pdb files from the EVFold run (http://evfold.org/evfold-web/evfold.do) (see references [96–98])

abolishes the GABA-inhibited anion flux [18]. A list of other common antagonists/antagonists of $GABA_A$ receptors and GABA-shunt modulators is summarised in Table 5, such as picrotoxin [116], benzodiazepines [117] and flumazenil [118]. Most of the agents listed in Table 5 are of plant origin and have not yet been tested on ALMTs or in plants. GABA-mediated effects have been observed in animals, fungi, and plants, and since many of the agents listed in Table 5 have been used in the characterisation of animal $GABA_A$ receptors, it would be instructive to test these in plants in regard to their mode of action on GABA-mediated regulation of anion channels and signalling in plants. If they also interact with the putative GABA-binding region in ALMTs, then it would appear that they may also have a biological function in plants; it is tempting to speculate that this has been recruited by the medical industry to act on equivalent sites in humans. The alternative hypothesis about the origin of these compounds is that they are synthesised by non-animal systems to act as defence or beneficial compounds. For instance, muscimol, derived from the mychorrhyzal fungi *Amanita muscaria,* can act as an insecticide by overloading the nervous system of insects. The decaying insects can then be used as a nutrient source for further fungal growth [119].

Link between aluminium, GABA, and calcium in animals and plants

It is perhaps a fascinating coincidence that in both animals and plants, there is interplay between Al^{3+}, Ca^{2+}, and GABA on certain transport proteins and that this has consequences for the development and growth of the organism. Al^{3+} is one of the most abundant metals on earth and found in most tissues, but is without an attributed beneficial physiological function [120]. In fact, Al^{3+} is associated with toxicity in both animals [121, 122] and plants [123–125]. In animals, accumulation within tissues causes various cognitive as well as physiological impairments [126–129] and in plants exposure to Al^{3+} causes inhibition of root growth, cytotoxicity, and decrease in yield on acidic soils [130–132]. Furthermore, in plants, Al^{3+} can inhibit some voltage-gated channels and glutamate receptor-mediated currents [133, 134]. In humans, Al^{3+} toxicity leads to conditions, such as dementia, Alzheimers, and Parkinsons [135, 136]. Aluminium has been shown to potentiate currents evoked by GABA in rat olfactory bulb mitral/tufted neurons [129] but had no effect on membrane currents induced by glutamate, glycine, *N*-methyl-D-aspartate, or kainate. It has been suggested that the $GABA_A$ receptors express two allosteric sites for Al^{3+}: one a high-affinity-binding site (potentiating) and the other a low-affinity-binding site (inhibiting) and the effect of Al^{3+} further depends on the subunit composition of the receptors. In adult male albino rats either fed with $Al_2(SO_4)_3$ in different doses or untreated, the levels of glutamate and glutamine increased in a dose-dependent manner in the brain tissue, while the GABA levels decreased [137] compared to controls. The mechanisms by which Al^{3+} causes changes in glutamate, glutamine, or GABA levels in brain is not very clear and one hypothesis is that Al^{3+} may induce modifications in the enzymes of the GABA shunt leading to neurotoxicity and neuropathology.

In plants, it is well known that Al^{3+} causes rhizotoxicity and impairs root growth and overall yield of plants in acidic soils [124, 138]. TaALMT1 confers Al^{3+} tolerance in wheat roots through Al^{3+} ion-activating TaALMT1 causing the release of malate that complexes the external Al^{3+} [139]. GABA inhibition of TaALMT1 modulates malate efflux through the channel. GABA is synthesised in the cytoplasm and enters mitochondria via GABA permease [140], but inhibition of malate efflux suggests that GABA signalling occurs in the apoplast. The question then arises as to how GABA enters the apoplast and exits the cell [27]. GABA is taken up into the cells via the high-affinity GABA uptake transporter GAT1 [141] and is then perhaps regulated by signalling in the cell via regulation of GAT1 and other GABA transporter/s. Interestingly, no GABA efflux transporter has been identified to date. Unlike animal systems [142], there is little information or experimental evidence on Ca^{2+} regulation of GAT1 from *Arabidopsis*. The expression of 7 of the 9, 14-3-3 genes identified in *Arabidopsis* seedlings is downregulated by GABA (10 mM) in the presence of high Ca^{2+} (22 mM) and requires functional ethylene and ABA signalling pathways [53], while low Ca^{2+} (2 mM) did not affect the transcripts. It would be interesting to study the expression of GABA shunt genes and ALMTs in root tips in the presence and absence of different concentrations of Ca^{2+}, Al^{3+}, and exogenous GABA to understand if there is an interaction between all three similar to animals.

Evolutionary insights into ALMTs and $GABA_A$ receptors

Gene and genome duplication has been documented as one of the most important factors in the evolution of eukaryotic animals and plants [143–145]. Gene duplication followed by gene divergence is thought to be the underlying factor in evolution of central nervous system in vertebrates [146]. Both the cationic (acetylcholine and serotonin) and anionic (e.g., GABA and glycine) ligand-gated channels have been predicted to have diverged before the origin of eukaryotes [147]. Despite this, plants do not possess any orthologous proteins to the mammalian GABA receptors, suggesting that ALMTs may have evolved convergently to fulfil a GABA-signalling role. $GABA_A$ receptors are made up of multiple subunits and 14 of the human $GABA_A$ receptor genes cluster on four chromosomes [148, 149]. Two clusters contain two genes encoding α, one gene encoding β and γ subunits each, while the other two clusters contain genes encoding α, β, γ, and ε subunits [148]. Evidence suggests that the four clusters arose from the duplications of and within a single $GABA_A$ receptor gene cluster with α, β, and γ subunits encoded for by single genes [148, 149]. It is thought that ε and π subunits also arose from gene duplications but not from the same four clusters [149]. Furthermore, an ancestral $GABA_A$ receptor gave rise to two monophyletic clades: one that has subunits that are involved in binding to benzodiazepines (α, ε, and γ) and the other that is not involved in binding to benzodiazepines (ρ, β, Δ, θ, and π) and this divergence occurred before the split from urochordates [150].

Whole genome duplications are thought to be the main source of gene duplications in plants, although individual gene duplications are also common [151]. It is thought that numerous genome duplication events have occurred during the diversification of angiosperms, including polyploidy [152–155]. Phylogenetic analyses of ALMT proteins from plants, such as *A. thaliana*, *P. trichocarpa*, *O. sativa*, *S. moellendorffii,* and moss *P. patens* subdivided these

Table 5 Overview of drugs tested as agonists, antagonists, or modulators of GABA receptors in animals and plants

Drug	Source	Action on animal GABA receptors	Effect on animal GABA receptors	References	Tested in plants	Effects in plants	References
Bicuculline	*Dicentra cucullaria; Corydalis* sp., *Adlumia* sp.	Competitive antagonist	Mimics epilepsy	[290]	Yes	Ameliorates the inhibition of anion flux by GABA	[18]
Picrotoxin	*Anamirta cocculus*	Non-competitive antagonist	Blocker for the $GABA_A$ receptor	[290]	No	Unknown	
Bilobalide and Ginkgolides	*Gingko biloba*	Negative allosteric modulator	Acts on $GABA_A$ receptors and $GABA_A$-rho receptors	[290, 291]	No	Unknown	
Muscimol	*Amanita muscaria*	Agonist	Sedative-hypnotic and dissociative psychoactivity	[292, 293]	Yes	Inhibits anion flux	[18]
GABA	Plants, chocolate, tea wine	Agonist	Reducing neuronal excitability	[110, 294]	Yes	Inhibits anion flux	[18]
Flavanoids	Red wine, vegetables, green tea	Modulators-Benzodiazepine binding	Anti allergic/anti inflammatory, anti microbial/ anti oxidant	[291]	No	Unknown	
α pyrones	*P. methysticum*, cinnamon, cloves, and ginger,	Positive modulators	Facilitates cell to cell communication	[291]	No	Unknown	
Apigenin	Matricaria *recutita* (Chamomile), parsley, celery, celeriac	Anxiolytic properties	Possible chemo-preventive role in Leukemia	[295–297]	No	Unknown	
Flumazenil	Synthetic	Benzodiazepine receptor antagonist	Anaesthesia reversal benzodiazepine overdose	[298, 299]	No	Unknown	
Amentoflavone	St. John's wart *Gingko biloba*	influences G-protein-coupled receptors, for serotonin, dopamine etc.	Anti cancer/anti malarial	[300]	No	Unknown	
Baclofen	Synthetic	Mainly $GABA_A$ receptor agonist	Spasticity/addiction	[41, 301, 302]	Yes	Increased GABA-mediated promotion of growth in *Lemna minor*	[22]
Gabaculine	*Streptomyces toyacaensis*	Irreversible GABA-α Ketoglutaric acid Transaminase inhibitor, GABA reuptake inhibitor	Research only purposes—increases GABA levels	[303, 304]	No	Unknown	
Vigabatrin	Synthetic	GABA-T inhibitor	Treatment of epilepsy	[111, 305, 306]	Yes	Increases endogenous GABA concentrations	[18]

Table 5 continued

Drug	Source	Action on animal GABA receptors	Effect on animal GABA receptors	References	Tested in plants	Effects in plants	References
GHB (γ-hydroxybutyric acid)	Endogenous—plants and animals	Naturally occurring neurotransmitter	General anaesthetic, insomnia, narcolepsy, alcoholism, recreational drug etc.	[307–310]	No	Unknown	
Barbiturates	Synthetic	Central nervous system depressants	Anxiolytic, sedative, hypnotic	[311–313]	No	Unknown	
Benzodiazepines	Synthetic	Inhibit $GABA_A$ receptors	Anxiolytic, sedative, hypnotic, muscle relaxant	[314, 315]	No	Unknown	

proteins into five distinct clades [38]. The ALMT family was initially thought to be specific to angiosperms, but, now, it has been shown that ALMTs are present in Bryophyta (mosses) and Lycophyta [58] and possibly algae [156]. Interestingly, no ALMTs have so far been identified in bacteria, fungi, humans, or amoeba, though the ALMTs share a domain of similarity to the fusaric acid resistance protein (FusC) effluxers in bacteria [157]. Phylogenetic analyses of 400 non-redundant ALMT proteins identified from 30 embryophyte species and 2 chlorophytes revealed that all belonged to a single group of orthologs indicating that they arose from a single ALMT-type protein [58]. However, it was observed that ALMT proteins from *S. moellendorffi* and *P. patens* formed two distinct groups in addition to the five clades identified [38, 58]. Furthermore, the different clades/groups arose by several gene duplication events in different lineages and underwent functional diversification, e.g., ALMTs from *Arabidopsis* [38]. When an in silico analysis of 116 ALMTs was carried out for the GABA motif from ALMTs from plants, it was observed that there were natural variants (Cys for Phe) in the amino-acid residue(s) that appear to be important for GABA binding [18]. This would potentially render such variants insensitive to GABA, but so far these have not been examined. Given the structural and functional diversity of full-length ALMT proteins, we performed a phylogeny of the amino-acid motif important for GABA binding from the ALMTs used by Dreyer et al. [58] in their phylogenetic analyses and also wheat (*T. aestivum*), barley (*Hordeum vulgare*), and rice (*Oryza sativa*) (Fig. 5) [58]. The motif for $GABA_A$ receptor from rat was used as an outgroup. It is interesting to note that the motif region from different ALMTs fall into similar clades identified for the full-length proteins [58]. The motif region from TaALMT1 from wheat, HvALMT1 from barley, and OsALMT5 from rice fall into the evolutionary clade 1 with ALMTs 1, 2, 7, and 8 from *A. thaliana*. It is interesting that ALMTs from wheat (TaALMT1), barley (HvALMT1), rice (OsALMT5), and *Arabidopsis* (AtALMT1) have been shown to be regulated by GABA [18], localised to the plasma membrane of either the root tips or guard cells. Not much is known about the transport characteristics of the other members of clade 1. The OsALMT9 from rice falls into the evolutionary clade 3 along with *Arabidopsis* ALMT12, 13, and 14, and it is interesting that all these members characterised so far localise to the guard cells and with the exception of AtALMT12 have been shown to be GABA sensitive [18]. Based on consensus sequence, sequence logo and residues in positions 3–5 (from L to R—presence or absence of F residue) for each clade (Fig. 5), clade 1 (except *P. trichocarpa* 006s21960.1, 0016s07070.1, and 009s02300.1—have two Cys in position 3 and 5), 2a, 3, 4, and 5—all have ALMTs that can be predicted to be GABA sensitive. Interestingly, clade 2 in this analysis splits into two parts—a and b (in comparison with phylogenetic analysis by Dreyer et al. 2012). Clade 2a has ALMTs that are predicted to be GABA sensitive, while clade 2b has those that may be weakly sensitive to GABA based on the fact that positions 3–5 have no F except *P. trichocarpa* 0010s13750.1. However, one must keep in mind that this prediction is based on the analysis of one GABA motif identified so far in ALMT1 proteins [18] and there is a possibility of more than one GABA motif occurring in the ALMTs.

GABA regulation in plants

GABA regulates plant growth and development

The comparison above between plant ALMTs and mammalian $GABA_A$ receptors indicates that ALMTs may respond to GABA in an analogous manner to that of GABA

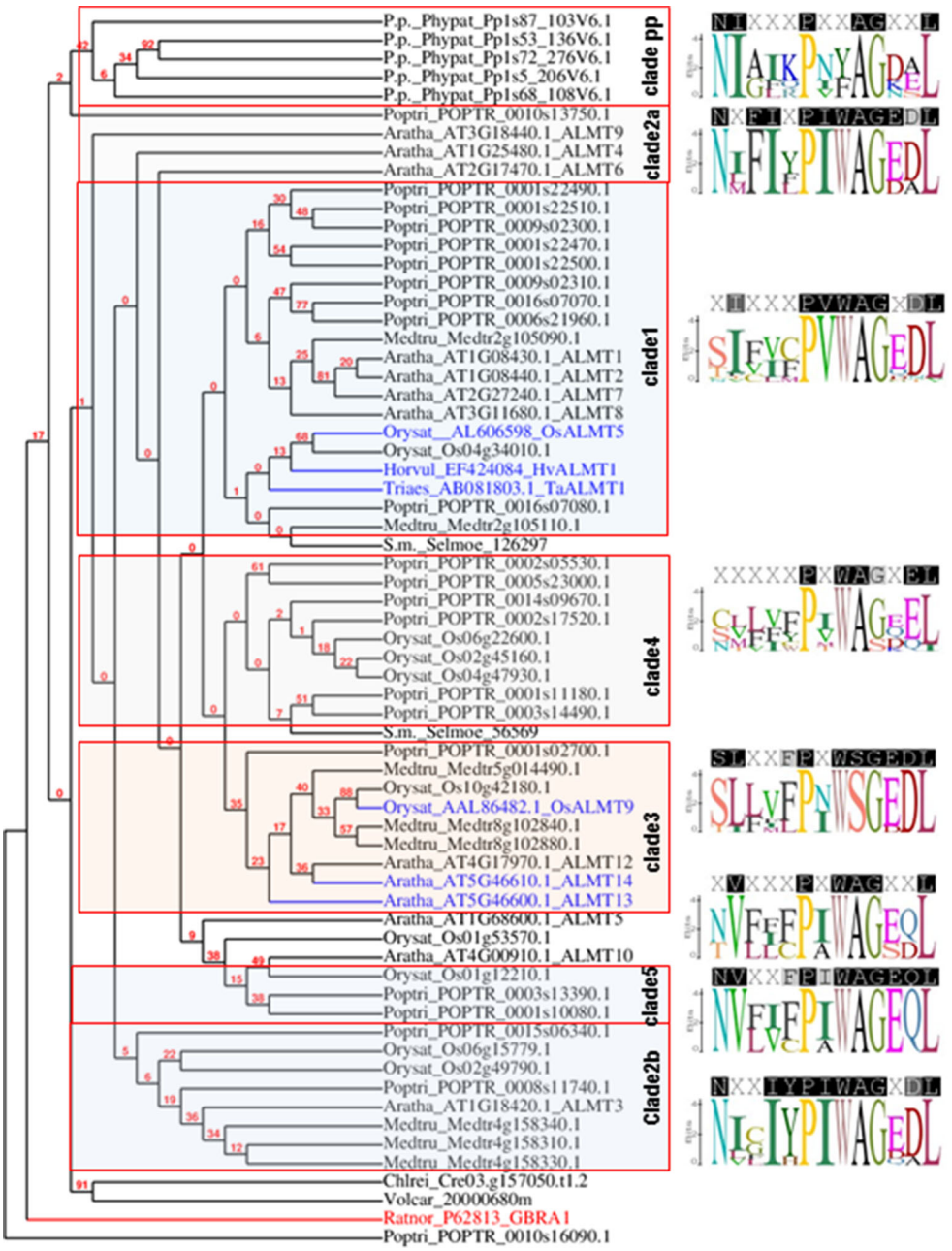

Fig. 5 Phylogenetic analyses of amino-acid residues important for GABA binding from ALMTs in plants. The full-length amino-acid sequences of ALMTs from *A. thaliana, P. patens, Poplar, M. truncatula, O. sativa*, *S. mollendorfii*, *T. aestivum, C. reinhardtii*, *V. carteri,* and $GABA_A$ α subunit from *Rattus novergicus* (rat) were aligned with MUSCLE. The region with residues important for GABA binding was extracted from the alignment and subjected to PhyML analysis at Phylogeny.fr program with bootstrapping procedure (100). The clade information has been overlaid from Dreyer et al. [58]. The sequence alignment was used to generate consensus sequence and sequence logo using Geneious 9.0.4. The *scale bar* to the *left* of the graph shows minimum and maximum coverages for the alignment, as well as a *tick* somewhere in between for the mean coverage. The height of the logo at each site is equal to the total information at that site, and the height of each *symbol* in the logo is proportional to its contribution to the information content

receptors, although the classification of ALMTs as a "GABA receptor" has not been thoroughly confirmed [18]. Current evidence proposes that GABA can act as a signal molecule in plants and aspects of this evidence will be further discussed below to explore how GABA is regulated by and/or modulates physiological process in plants.

A seminal paper for plant GABA research in the 1990s discovered that GABA can impact plant growth and development [158]. The overexpression in tobacco (*Nicotiana tabacum*) of a *GAD* from petunia, with its C-terminal calmodulin-binding domain removed to make it constitutively active, increased the tissue GABA concentration above wildtype levels and caused slow growth, and more branched and shorter cortical parenchyma cell elongation [158]. Physiological evidence for the presence of GABA receptors in plants was first observed in duckweed (*Lemna minor* L.) [22], where, in the presence of 5 mM GABA and nutrient solutions, a two–threefold increase in plant growth was observed over that cultured in nutrient solution alone, and the addition of 0.5 mM 2-aminobutyric acid inhibited growth compared to control plants. This is in contrast to the GABA inhibition of growth observed in tobacco [158] and soybean hypocotyl tissue [159]. In sunflower, the effect of GABA was dose dependent with low concentrations promoting growth and high concentrations inhibiting growth [160]. Cell elongation was severely impaired in

Arabidopsis pollen tubes, primary root, and hypocotyls when the GABA transaminase (*GABA-T*) gene was disrupted leading to elevated tissue GABA concentrations [19, 161, 162]. Exposure to 10 mM GABA, further increased tissue GABA concentrations in a *GABA-T* T-DNA insertion line (named *gaba-t* or *pop2*) [19, 162]. As multiple stresses increase GABA concentration in tissues—as has been well documented [22]—these effects of GABA detailed above have been proposed to mimic the impact of stress on growth and development [163]. Besides, there is evidence that GABA regulates other processes not associated with stress, such as the possible regulation of nitrate uptake in *Arabidopsis* and *Brassica napus* [164–166], nodule formation in *Medicago* [167–169], and control of leaf senescence in *Arabidopsis* [170]. Endogenous GABA concentrations exhibit a light-rhythm-dependent oscillation in *Arabidopsis* tissue [171], suggesting that GABA might be involved in regulation of, or regulated by, the plant circadian clock. GABA may also be involved in long-distance transport via xylem and phloem in plants (see "Is GABA involved in long-distance transport regulation?").

The first piece of substantive evidence for a signalling role of GABA in plants was that it affected pistil–pollen tube communication [19, 20]. GABA has a biphasic effect on pollen tube growth. At low concentrations, it increased growth rate in vitro, whereas at concentrations, greater than 1 mM pollen tube growth rate was retarded [20]. A gradient of GABA (in the micromolar range, Table 1) increasing from the stigmatic surface toward the ovary was proposed to guide pollen tubes in tobacco to optimize fertilization. When GABA was constitutively high, pollen growth was aberrant and fewer ovules were fertilised [19, 162]. GABA regulation of pollen tubes is widespread across the plant kingdom with effects observed for both angiosperms and gymnosperms [18, 172]. Pollen germination and polarization of *Picea wilsonii* is affected by GABA, supplementation with GABA between 50 and 100 mM promoting pollen tube elongation, while supply with higher than 100 mM or with lower than normal levels of GABA (via 3-MPA treatment) severely reduced pollen germination and tube growth [49, 173]. Pollen tube growth of both *Arabidopsis* and grapevine was also found to be inhibited by muscimol (an agonist of $GABA_A$ receptor) and this affect was antagonized by bicuculline (a competitive antagonist of GABA) [18]. Since 3-MPA, muscimol, and bicuculline are agents commonly used for GABA-receptor diagnostics in mammals [40], the observed change in pollen tip growth by these may involve an alteration of GABA-mediated ion flux across its cell membrane [18, 60]. Although the mechanism by which GABA regulates tip growth is not yet ascertained, it is possible that ALMTs and/or other targets are situated in the pollen tube plasma membrane.

Tip growth of pollen is dependent upon oscillations in ion influx (e.g., Ca^{2+}) and efflux (e.g., Cl^-) across the plasma membrane that drives oscillations in cytosolic ion concentrations [49, 60, 173–175]. It was observed that 1 μM GABA increased cytosolic Ca^{2+} in *N. tabacum* pollen protoplasts [20], and 1 mM GABA elicited a Ca^{2+} influx into pollen tubes through a pathway independent of glutamate-induced increases in cytosolic Ca^{2+} (1 μM was not tested in this case) [20]. Patch clamp electrophysiology found that low millimolar (e.g., 1 mM) GABA increased inward currents, which in the conditions used could have been either anion efflux or Ca^{2+} influx, whereas these currents were inhibited by 100 mM GABA [20]. As GAD is activated by increases in cytosolic Ca^{2+}, GABA-induced Ca^{2+} influx will potentially affect production of GABA and feedback on ion flux across the membrane that may modulate pollen tube growth [20].

GABA regulates plant abiotic stress responses

Diverse abiotic stresses drive GABA accumulation in plants, including salt, anoxia, hypoxia, heat, mechanical damage, drought, cold, and waterlogging, but the speed of the GABA increase varies from seconds to a few days [176], reviewed in [18, 177–188]. Amongst these stresses, salt-induced GABA accumulation has been studied most broadly in terms of the number of plant species, including alfalfa (*Medicago sativa* L.), *Arabidopsis*, barley, tobacco, *Populus* × *canescens*, rice, and soybean [161, 177, 180, 189–192]. However, the molecular mechanism behind the GABA increases and its consequences has only been probed in *Arabidopsis* [161, 177, 180, 189–192]. The *Arabidopsis* seedling produced ~15 μmol g^{-1} DW level of GABA under 150 mM salt stress in shoots, this was approximately 20-fold higher than in non-stressed conditions (0.7 μmol.g^{-1} DW) [161]. The *Arabidopsis* GABA transaminase (*GABA-T*) mutant (*gaba-t* or *pop2),* which blocks GABA catabolism and causes GABA accumulation (see "Elements that shape GABA signals in plants via the GABA shunt"), is more sensitive to salt stress, as indicated by primary root growth being inhibited by 17% by 150 mM NaCl through reduced cell elongation compared to that of wild type [161]. The investigation of global transcriptional profile found that the *pop2* mutant lines had ten cell-wall related (four upregulated and six downregulated), eight carbon metabolism (upregulated), and three polyamines metabolism genes differentially expressed, consistent with metabolomics analysis showing that central carbon metabolism was disrupted by salt stress [193]. Many of these genes were also regulated by application of 10 mM GABA to *pop2* plants independent of salt stress, indicating that GABA plays a key role in the response to salt [162]. Thus, it was proposed

that GABA-mediated response to salt stress involves regulation of central carbon metabolism and cell-wall modification [161, 193]. Intriguingly, the disruption of vacuolar *AtALMT9* resulted in low sodium and chloride accumulation in shoots [194]; however, no study has suggested any correlation of GABA with ALMTs in such salt response.

Drought stress was reported to promote GABA synthesis in *Arabidopsis*, soybean, sesame (*Sesamum indicum* L.), bean (*Phaseolus vulgaris* L. cv. Topc rop), and turnips (*Brassica rapa* L. var. Shogoin) [181, 188, 195–197]. The disruption of glutamate decarboxylase (*GAD1* and *GAD2)* genes depleted GABA production in *Arabidopsis* T-DNA insertion line *gad1/gad2* and this increased stomatal conductance and made them more sensitive to drought [188]. The triple mutant *gad1/gad2* × *pop2–5* increased endogenous GABA production and rescued the drought sensitive phenotype of *gad1/gad2* and recovered stomatal conductance to wild-type levels [188, 198]. Therefore, GABA appears to regulate plant gas exchange [188]. Nevertheless, there has been no evidence presented so far to determine whether GABA regulates any ion channels or transporters involved in stomatal opening or closure (e.g., AtALMT12) [188].

Another study that correlates GABA metabolism with plant hypoxic response was demonstrated in *Prunus persica* [199]. The exogenous application of GABA increased tissue GABA concentration and improved the performance of hypoxia-sensitive *Prunus* genotype under waterlogging stress, this included a higher stomatal conductance, lower H_2O_2 production, and less leaf lesion; whereas there was no further improvement to the hypoxia-tolerant genotype [199]. This implicates that the upregulation of GABA production may positively contribute to plant response to hypoxia stress, although the molecular mechanism behind this is not yet clear.

GABA regulates Al^{3+}tolerance in plants

A common problem in acidic soils is that Al^{3+} becomes soluble in the soil solution. In wheat, two near-isogenic lines (NILs)—ET8 (Al^{3+} tolerant) and ES8 (Al^{3+} sensitive)—were first isolated at a single locus designated as *Alt1,* essential for root Al^{3+} sensitivity by Delhaize et al. [200, 201]. Later, the gene *TaALMT1* was identified as underpinning the locus *Alt1* as the protein that facilitates malate efflux from root tips, which chelates Al^{3+} and prevents Al^{3+}-inhibition of root growth. The high expression of *TaALMT1* in ET8 compared to ES8 is believed to confer the difference in Al^{3+} sensitivity between the two NILs [202]. Interestingly, the Al^{3+} sensitivity of ES8 could be phenocopied in ET8 via the exogenous application of GABA or muscimol [18]. GABA production is induced under acidic conditions; however, it was found that under acidic conditions, such as when Al^{3+} was present, GABA concentrations were lower in the root tips of ET8 compared to when Al^{3+} was absent, and this coincided with the induction of malate efflux [18]. Treatment with GABA inhibited malate efflux under these conditions and abolished Al^{3+} tolerance in roots [18]. The down regulation of GABA is essential for plant adaptation to acidic (Al^{3+}) stress. This led to the discovery that TaALMT1, and other ALMTs more broadly can have their transport activity regulated by GABA [18]. Notably, GABA (~2 μM) was previously found as one predominant molecule in root exudates (followed by putrescine, alanine, betaine and glutamate) at near neutral pH (6.5–6.8) [203]. It has been suggested that wheat can reuptake a range of organic nitrogen compounds at sub-micromolar concentrations from root exudates. A number of transporters have been identified to be involved in secretion of root exudates [204, 205], but to date, the mechanism of GABA efflux from roots has not been identified.

GABA regulates plant defence

GABA rapidly accumulates in the apoplasm following herbivory attack and pathogen infection and it is used in defence responses, and possibly signalling [198, 206–210]. The rapid increase in GABA by 5-fold in tobacco was detected within 10 min of the leaf being crawled upon by the tobacco budworm (*Heliothis virescens*) and by 11-fold in soybean following leaves being crawled upon by *Choristoneura rosaceana* cv Harris larvae [211]. Transgenic tobacco plants overexpressing a petunia *GAD* gene achieved a higher tissue GABA concentration and conferred more resistance to *Meloidogyne hapla* than wild-type plants with significantly fewer egg masses on the root surface by ≥50% [207]. The triple mutant *gad1/gad2* × *pop2–5* line had a greater GABA content within tissue and a greater resistance against insect herbivores *S. littoralis* than wildtype *Arabidopsis* [198]. These observations point to a positive correlation between GABA induction and herbivory defence [208]. This GABA increase is considered to cause physiological disorders to insect larvae via the inhibition of their neuronal GABA-targeted Cl^- channels that results in a reduced growth and survival rate [208, 212–216].

In plant-microbial interactions, GABA is also induced and has a positive contribution to plant defence against microbial invasion. The application of cell-wall elicitor derived from rice blast fungus (*Magnaporthe grisea*) increased GABA content by 12.5-fold in rice suspension cultured cells [209]. Exogenous application of GABA enhanced the resistance of tomato to *Botrytis cinerea* [217]. To further explore the GABA correlation with pathogen

defence, Park and co-workers (2010) deleted three GABA transaminase genes (*GabT*) in *Pseudomonas syringae* DC3000 to generate a triple mutant strain –*ΔgabT2/T3/T1* with a defect in GABA degradation activity resulting in approximately 2.5-fold higher levels of GABA than in wild type. This mutant *P. syringae* strain *ΔgabT2/T3/T1* weakened its infection on *Arabidopsis* leaves, and following a disruption of *GABA-T* in *pop2* mutants from *Arabidopsis*, *ΔgabT2/T3/T1* displayed further reduced colonization [210]. This advocates that pathogen induced GABA production by plants, on the one hand, is positively correlated with its microbial resistance, while on the other hand, the ability of a pathogen to metabolize GABA is associated with their infection capacity. The mechanism behind GABA-mediated defence against *P. syringae* is unclear; however, we can see some hints from plant interaction with *Agrobacterium tumefaciens* [218–220]. *A. tumefaciens* produces crown galls on infection, and the level of quorum-sensing signal [*N*-(3-oxoctanoyl) homoserine lactone-OC8HSL] was inactivated by GABA [218]. Two GABA-binding proteins have been identified from *A. tumefaciens*—the non-selective GABA sensor Atu2422 (binding to a board spectrum of amino acids) and the selective GABA sensor Atu4243, both of which are critical for the inactivation of OC8HSL quorum-sensing signal [220, 221]. An analysis of Atu4243 crystal structure identified serial conserved residues for GABA interaction ($W^{8}T^{12}E^{60}F^{99}Y^{101}W^{200}R^{203}D^{226}Y^{262}$), which is also possessed by *P. syringae* ($W^{8}T^{12}E^{60}F^{99}F^{101}W^{200}R^{203}D^{226}Y^{262}$) [220], implicating that plants may have similar machinery for GABA-mediated defence against both *A. tumefaciens* and *P. syringae*. Intriguingly, these key GABA-interaction residues from Atu4243 do not appear in the plant or animal GABA-regulated region (as reviewed in "Structure and topology of plant ALMTs vs. mammalian GABAA"). So far, however, no evidence is available to indicate any GABA-regulated ion flux or channel is involved in this plant-microbial interaction.

Crosstalk between GABA and other signalling molecules/hormones

GABA has been proposed to be a stress-related metabolite with links to plant hormones [22, 222–225] and the oxidative burst [180, 183, 226–228]. Exogenous GABA has been reported to promote ethylene synthesis in sunflower and *Stellaria longipes* [160, 229]; however, it reduced ethylene production in *Caragana intermedia* roots under salt stress [230]. Alternatively, perturbed ethylene levels also impairs GABA metabolism in plants. The exogenous application of ethylene inhibitor (aminoethoxyvinylglycine, AVG) decreased GABA accumulation in Creeping bentgrass (*Agrostis stolonifera*) (cv. Penncross) under heat stress [225]. The ethylene inhibitors AVG and AIB (amino isobutyric acid) promoted Al^{3+}-activated malate efflux from the root tips of wheat ET8 line [231], while ethylene donor (Ethrel) inhibited Al^{3+} induced efflux from tobacco cells when expressing *TaALMT1* [231]. Coupling with the evidence that Al^{3+} stress reduces endogenous GABA production leading to increased malate efflux [18] (as discussed above), we speculate that the application of ethylene inhibitor somehow modulates GABA concentrations or perhaps ALMT expression to maximise malate efflux [18, 231]. There may also be a cross talk between GABA and ethylene that confers a negative regulation of malate efflux, perhaps via regulation of TaALMT1 activity.

Other hormones can also affect GABA metabolism in plants. A T-DNA insertion into the *NCED3* (*9-cis-epoxycarotenoiddioxygenase 3*) gene in *Arabidopsis* impaired dehydration-induced abscisic acid (ABA) synthesis [223, 232, 233] and the mutant had a significantly higher GABA accumulation compared to wild type [223]. An overexpression of two DELLA subfamily members—*gibberellins (GAs) insensitive* gene (*GAI*)—and repressor of *GA1*-like (*RGL1*) in *Populus* seedlings increased GAs level by 12- and 64-fold, respectively; while GABA was also threefold higher in these transgenic seedlings compared to wild type [222]. Chilling treatments were found to increase GABA content in loquat fruit, and this GABA was further increased when methyl jasmonate (MeJA) was applied in addition to chilling [234]. However, its role in plant-herbivory interaction was not tested, although both GABA and MeJA appear to contribute to plant defence against herbivory attack [198, 235]. In *Arabidopsis*, the triple T-DNA insertional mutant of *GAD1*, *GAD2,* and *GABA-T* (*gad1/gad2* × *pop2* line) over accumulated GABA and displayed better systemic defence against the insect herbivore *Spodoptera littoralis* [198], whereas the levels of defence hormone against *S. littoralis*—jasmonate (JA) and its bioactive derivative, (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) showed no obvious difference [198, 236]. GABA may thus interplay with plant hormones, such as ABA, GAs, and JA, but possibly only upon certain stimuli (e.g., JA). Taken together, an interaction between GABA metabolism and hormone production is likely to modulate several physiological processes in plants and requires further research.

Apart from hormones, GABA metabolism has also been shown to have links with ROS production (e.g., H_2O_2). When *C. intermedia* was grown in 300 mM NaCl, endogenous H_2O_2 gradually increased in root and shoot tissue for up to 72 h [230]. However, this was abolished by exogenous application of GABA [230]. Similarly, potassium cyanide treatment, which mimics hypoxia stress, stimulated H_2O_2 production in grapevine buds, and again,

this was reduced by GABA [186]; this hypoxia-induced H_2O_2 production was also lowered by exogenous GABA treatment in *Prunus* [199]. Elicitors from rice blast fungus (*Magnaporthe grisea*) increased GABA production and decreased GABA-T activity, and the activation of ROS scavenging recovered the GABA-T activity in this case [209]. Thus, GABA was proposed to protect plants from oxidative stresses [16, 206].

Cytosolic pH modulates ALMT activity

A model has been recently proposed by Gilliham and Tyerman [27] for regulation of plasma membrane ALMT activity by malate and GABA, which, respectively, contributes to a positive and a negative regulation of TaALMT1 activity. This confers a connection between metabolism and membrane signalling [27]. On the one hand, malate is a metabolite regulated by cytosolic pH: (1) high cytosolic pH increases malate synthesis as it consumes OH^- and (2) low cytosolic pH inhibits malate synthesis and stimulates its metabolism into pyruvate together with CO_2 and OH^- release [237]. On the other hand, GABA is also regulated by cytosolic pH [16]: (1) the acidic pH stimulates the synthesis of GABA (via up regulation of GAD activity) and (2) this process is reversible when increasing cytosolic pH [238, 239]. Taken together, it appears that high cytosolic pH stimulates malate production and suppresses GABA leading to a relatively low GABA-to-malate ratio, and likely a high ALMT activity, whereas cytosolic acidification will shift to a higher GABA-to-malate ratio that reduces activity of plasma membrane ALMTs. Therefore, changes in cytosolic pH induced by stresses (e.g., salt and hypoxia) possibly alters ALMT activity, and then changes in cell membrane voltage and transport to elicit downstream response [240, 241].

The GABA-malate connection at the tonoplast

The model proposed by Gilliham and Tyerman [27] as described in the section above connects the GABA-malate metabolism to the plasma membrane signal mediated by ALMTs. In fact, a number of ALMT family members (e.g., AtALMT6 and VvALMT9) are also targeted to the tonoplast membrane [56, 57, 61, 242]. For instance, *ALMT9* from grapevine encodes a vacuolar membrane malate channel sensitive to GABA at high affinity (6 μM) when expressed in *X. laevis* oocytes [18, 61]. Presumably, the model proposed by Gilliham and Tyerman [27] on plasma membrane regarding the ALMT-mediated GABA-malate signalling paradigm could be mirrored at the tonoplast [27]. Thus, stress-induced GABA elevation in the cytoplasm could transiently increase the cytosolic GABA:malate ratio to negatively modulate tonoplast ALMT activity and reduce malate release from cytoplasm into vacuoles. This will also lead to a change in vacuolar membrane potential and perhaps other ion fluxes across the tonoplast. The tonoplast localised GABA transporters, such as the cationic amino-acid transporters (CATs) from *Solanum lycopersicum* (SlCAT9) catalyse GABA uptake into vacuoles [243], and may have a similar role to that of GAT1 in this model.

Is GABA involved in long-distance transport regulation?

A range of signalling molecules can be translocated between shoot and root via the plant vascular system, including hormones, ROS, and salicylic acid (SA), as reviewed in [244, 245]. GABA has been found in the xylem sap of walnut [246] and salt treatment increases GABA in the root xylem of soybean [247]. Approximately 0.7 μmol g^{-1} GABA was present in soybean nitrogen-fixing nodules, however, only 0.01 μmol g^{-1} GABA and almost no GAD activity were detected in bacteroids of cowpea *Rhizobium* MNF2030, suggesting that GABA in the nodules was probably supplied by the host [167]. Artificial feeding of 15 mM GABA to *M. truncatula* petioles doubled GABA concentration in nodules, and enhanced nodule activity and N_2 fixation [248]. In this case, more GABA was likely transported into nodules and might be correlated with the observed increases in nodule activity and N_2 fixation. Nevertheless, it is uncertain whether this rapid change of GABA levels in nodules was due to translocation via xylem or phloem from one part of the plant to another, or due to de novo synthesis in response to stresses (e.g., wound) [249]. Therefore, whether GABA is involved in long-distance transport within plants still remains inconclusive and hard to probe [249]. The development of a fluorescence GABA sensor and its application to intact plants would be of benefit to such studies [250].

Elements that shape GABA signals in plants via the GABA shunt

In mammalian neuron cells, a GABA signal is generated via GABA synthesis in presynaptic cells from Glu catalyzed by two GAD enzymes, GAD65 and GAD67 [251]. GABA is then transported via vesicles by a vesicular neurotransmitter transporter (VGAT) [252] and released into the extracellular space for activation of GABA receptors and inhibitory neuron signal transmission. The GABA signal is terminated via reuptake by surrounding glial cells through plasma membrane GABA transporters (GATs) [253] and degraded by GABA-T [254, 255].

The enzymes engaged in the GABA shunt are conserved in both animal and plant kingdoms [13, 163]. GABA is synthesised from Glu in the cytoplasm by GADs with CO_2 release in plants [14] and mammals [158, 256]. The C terminus of GAD2 from *Arabidopsis* and rice contains an autoinhibitory CaM-binding region, the deletion of which increases GAD2 activity by 40-fold in rice and leads to GABA overproduction by 100-fold in seedlings [257]. In *Arabidopsis*, CaM T-DNA insertion mutant lines *cam1*, *cam4*, *cam5-4*, *cam6-1,* and *cam7-1* seedlings, there is significantly more GABA produced by H_2O_2 and paraquat treatments [183], so Ca^{2+}/CaM indirectly regulates GABA metabolism and GABA accumulation in plants [158, 163, 171, 258, 259]. GABA is taken up into mitochondria through a mitochondrial-localised GABA permease (GABP) [140] and catabolised by GABA-T into succinic semialdehyde (SSA) and finally succinate [14, 249, 260], this process is similar to the biological process in mammals [13]. In *Arabidopsis*, knocking out *GABA-T* (*pop2/gaba-t)* blocks GABA degradation resulting in more than tenfold GABA over accumulation [19, 161, 162, 198, 261]. Succinate semialdehyde (SSA) as the downstream metabolite of GABA is further catabolised into succinate by succinate semialdehyde dehydrogenase (SSADH) [260]. The disruption of this single *SSADH* gene in *Arabidopsis* causes necrosis, constant higher GABA, and H_2O_2 over accumulation, and leads to hypersensitivity to light and heat stress [24, 260, 262, 263]. In *ssadh* mutant lines, the hypersensitive phenotype is partially relieved by treatment with vigabatrin as an inhibitor of GABA-T and GABA degradation [260, 262, 263]. Interestingly, crossing *ssadh* with *pop2-4* generates *ssadh/pop2-4* line that has higher GABA levels in tissue, rescues *ssadh* dwarf, and hypersensitive phenotypes, and with H_2O_2 production at basal levels similar to wild-type seedlings [264]. SSADH is also reported to control the robust leaf patterning and formation of the adaxial–abaxial axis of leaf primordia through a screening of *enlarged fil expression domain1* (*enf1*) mutant (*enf1* = *ssadh*) [265]. Vigabatrin has not been applied to test its effect on the *enf1* mutant, but the *enf1*/*gaba-t* (= *ssadh/pop2-4*) has a wildtype-like leaf patterning [265]. The manipulation of tissue GABA levels through a T-DNA insertional mutation of *Arabidopsis GABA-T*, *GAD,* and *SSADH* can be phenocopied in tomato via virus-induced silencing of their homologs from tomato (*SlGABA-Ts*, *SlGADs,* and *SlSSADHs,* respectively) [266]. A study led by Seher et al. has measured tissue Glu, GABA, succinate, and total nitrogen concentrations as well as glutamate dehydrogenase (GDH) and GAD activities in 16 different plant species. They found that a large variation in GAD and GDH activity appears between different plant species and this does not match their endogenous N, Glu, and GABA content [267]. Accordingly, the tissue GABA levels are not simply determined by one or two enzymes. It appears that GADs, GABA-T, SSADH, and GAD interact, with Ca^{2+}/CaMs impacting on GABA production in all cases. Their interaction, perhaps together with other elements, e.g., GABA transport and compartmentation, carbon metabolism via tricarboxylic acid cycle (TCA cycle) and malate [27, 62] coordinate the generation and/or termination of GABA signals. The perturbed tissue GABA levels via the manipulation of these GABA shunt elements has successfully impaired GABA-mediated signalling and helped us explore GABA metabolism and mediated signalling in plants. Nevertheless, these different elements essentially display differential cell-type expression patterns [19, 162, 265], thereby certain GABA signals may be shaped only in particular cell types. In this case, a cell-type modification of GABA shunt elements possibly causes a cell-specific GABA-signalling perturbation, which is necessary to dissect the GABA roles in different cell types and particular physiological processes.

In addition, the disruption of elements in the GABA shunt is not always associated with perturbed GABA concentrations but yet still alters plant growth, development, and stress responses [140, 268]. The T-DNA insertion into either *GABP1* or *GAT1* fails to change GABA levels in mutant tissue [140, 269]. Knocking out *GABP1* significantly reduces mitochondrial GABA uptake rate by >40% and lowers CO_2 evolution (approximately 20%), so that it impairs GABA flow into the TCA cycle and mitochondrial respiration [140]. The high-affinity GABA transporter, GAT1 localised at the plasma membrane is thought to only reuptake GABA into the cytoplasm [27, 268], reminiscent of mammalian GATs. As such supply of exogenous GABA does not increase tissue GABA level in the *gat1* mutant, the disruption of *GAT1* caused no change in tissue GABA levels but altered the metabolic carbon–nitrogen equilibrium and response to low-carbon and nitrogen-environment in plants (e.g., Glu, malate, fructose, etc.) [269]. These two cases indicate that the disruption of certain GABA shunt elements does not always alter GABA concentrations in plants; however, it may still impair GABA-associated physiological processes.

Conclusions and Future Research

The recent discovery of plant GABA-regulated ion channels—ALMTs—opens new pathways for GABA research in plant biology, and here, our review provides an insight into the similarity and differences between plant ALMTs and animal $GABA_A$ receptors, the molecular determinants of GABA regulation by ALMTs proteins, the connection between GABA metabolism with GABA-mediated ion flux and physiology, and elements shaping potential GABA

signals in plants. The comparison of literature from animals and plants suggests that common features exists in both, such as: (1) residues important for GABA sensitivity; (2) GABA regulation of anion flux; and (3) common drugs that modulate GABA receptor activity, as well as differences, such as (1) limited homology in predicted full-length amino-acid sequence of the $GABA_A$ receptor (similarity is restricted to a 12 amino-acid stretch); (2) topology—mammalian receptor has four transmembrane domains, while the plant ALMT has six (or more) predicted transmembrane domains; (3) mammalian receptor is heteropentamer, while nothing much is known in plants regarding the subunits, but we do know that plant receptor can function as a homomer, since the expression of only one gene is sufficient to elicit functional response to GABA; and (4) GABA-binding site in mammalian receptor is located at the N terminus, while the predicted-binding site in ALMT is located at the end of transmembrane 6. Interestingly, most of the drugs that are modulators of mammalian GABA receptors are of plant origin, and therefore, the application of these drugs could well interact with the predicted GABA-binding region in ALMTs and will help further elucidate the molecular identity and basis of GABA regulation of ion fluxes in plants.

The characterisation of the predicted GABA-binding motif in plants is still in its infancy, and there are key research gaps. It remains to be shown: (1) whether GABA binds to the identified aromatic amino-acid residues in ALMT1; (2) what residues line the binding site and the pore; (3) the kinetics of GABA binding; (4) whether there is more than one region in the ALMT proteins involved in GABA-mediated regulation; (5) whether there are other metabolites, such as amino acids and compounds, related to GABA metabolism that are involved in regulation of ALMTs/ion channels; and (6) what the tertiary structure is of ALMTs. In addition, a number of GABA-mediated physiological processes in plants may require the participation of ALMTs to transduce GABA metabolism into plasma- and/or tonoplast-membrane signalling. The interaction between GABA and other signalling molecules may also contribute to certain responses albeit the candidates remain elusive. GABA signals controlled by GABA shunt elements appear to be shaped in particular cell types, although it is still inconclusive whether GABA signals are involved in long-distance translocation within plants. However, the recent research on plant GABA highlighted in this review suggests that new insights into the GABA regulation of physiological, developmental, and growth processes in plants may rapidly occur in the near future.

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