

## SUBMITTED VERSION

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 **$\gamma$ -Aminobutyric acid (GABA) signalling in plants**  
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# Cellular and Molecular Life Sciences

## γ-aminobutyric acid (GABA) signalling in plants

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<b>Abstract:</b>	<p>The role of γ-Aminobutyric acid (GABA) as a signal in animals has been documented over the past 6 decades. 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 receptors in terms of their molecular identity, structure, 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, the possibility that there may be other GABA binding sites and regions in the ALMT proteins (eg amino acid residues such as arginine and tyrosine) and explore the potential interactions between GABA and other signalling molecules.</p>				
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1  **$\gamma$ -aminobutyric acid (GABA) signalling in plants**

2  
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11 **Abstract**

12  
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14 contrast, evidence that GABA is a signal in plants has only emerged in the last 15 years, and it was not until last  
15 year that a mechanism by which this could occur was identified – a plant ‘GABA receptor’ that inhibits anion  
16 passage through the Aluminium Activated Malate Transporter family of proteins (ALMTs). ALMTs are  
17 multigenic, expressed in different organs and present on different membranes. We propose GABA regulation of  
18 ALMT activity could function as a signal that modulates plant growth, development and stress response. In this  
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20 molecular identity, structure, mode of action and signalling roles. We also explore the implications of the  
21 discovery that GABA modulates anion flux in plants, its role in signal transduction for the regulation of plant  
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23 amino acid residues such as arginine and tyrosine) and explore the potential interactions between GABA and other  
24 signalling molecules.  
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	27	<b><u>Keywords</u></b>
1	28	$\gamma$ -aminobutyric acid
2	29	Aluminium-activated malate transporters
3	30	GABA <sub>A</sub> receptors
4	31	Signalling
5	32	GABA metabolism
6	33	Carbon nitrogen balance
7	34	Stress response
8	35	Topology
9	36	Pharmacology
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39 **Abbreviations**

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1		
2	3-MPA	3-mercaptopropionic acid
3	ALMT	Aluminium (Al <sup>3+</sup> )-activated malate transporter
4		
5	Cys	Cysteine
6	EC <sub>50</sub>	Half-maximal response
7	F / Phe	Phenylalanine
8		
9	GABA	γ-aminobutyric acid
10	GABA-T	GABA transaminase
11	GABP	GABA permease
12	GAD	Glutamate decarboxylase
13	GAT	GABA transporter
14	GDH	Glutamate dehydrogenase
15	E / Glu	Glutamic acid
16	I / Ile	Isoleucine
17		
18	SSA	Succinic semialdehyde
19	SSADH	Succinic semialdehyde dehydrogenase
20	T / Thr	Threonine
21	D / Asp	Aspartic acid
22	V / Val	Valine
23	Y / Tyr	Tyrosine
24	Q / Gln	Glutamine
25	L / Leu	Leucine
26	R / Arg	Arginine
27	TMDs	Transmembrane domains
28	K / Lys	Lysine
29	S / Ser	Serine
30	G / Gly	Glycine

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## 42 Introduction

43  
44 The non-proteinogenic amino acid  $\gamma$ -aminobutyric acid (GABA) was first isolated in 1949 from potato tubers [1],  
45 prior to its discovery in animal brain extracts [2]. Soon after, in the 1950s and 1960s, evidence was gathered that  
46 suggested GABA might act as an inhibitory neurotransmitter in animals; GABA was found to suppress impulses  
47 generated by crayfish stretch receptor neurons [3,4]. Yet, it was not until Bloom et al. (1971) that GABA was  
48 localised to mammalian nerve terminals [5], and it took a further ten years until the mechanism by which GABA  
49 acts as an inhibitory neurotransmitter was identified – via its activation of GABA<sub>A</sub> (ionotropic) and GABA<sub>B</sub>  
50 (metabotropic) receptors [6]. In mammals, GABA counteracts the action of excitatory neurotransmitters in  
51 the mature brain [5], through the activation of a Cl<sup>-</sup> conductance that passes through GABA<sub>A</sub> receptors into mature  
52 neurons leading to membrane hyperpolarisation [7]. This prevents the neurons from firing and thus has a calming  
53 effect [8]. Its action has been mainly described in the nervous system where GABA receptors regulate brain  
54 function and development [9,10], although GABAergic receptors have also been described as functioning in other  
55 tissues beyond neuronal cells, such as human organs [11,12]. This has been extensively reviewed by Owens and  
56 Kriegstein (2002) [10,13] GABA as a signalling molecule in animals has been studied over six decades, whereas  
57 in plants it is mostly defined as a carbon – nitrogen metabolite [14-16]. This said, various evidence has been  
58 mounting since the 1990's that GABA may act as a signal in plants, including: i) GABA concentration in plant  
59 tissue is variable (0.03 ~ 6  $\mu\text{mol g}^{-1}$  fresh weight) and prone to large and rapid increases (< thousand fold)  
60 following exposure to a multitude of biotic and abiotic stresses [17,18]; ii) GABA concentration gradients can be  
61 found in plant tissues [19,20]; iii) GABA metabolism is compartmentalised intra- and inter-cellularly [21]; iv)  
62 GABA and GABA receptor agonists and antagonists alter plant growth[22]; v) GABA binding sites have been  
63 detected on plant cell membranes [20,23] and recently, vi) the identification of GABA-regulated ion channels in  
64 plants that also have their activity regulated by drugs known to affect GABA receptors in animals [18].  
65

66 A number of reviews have been published in the past two decades, which have summarised plant GABA  
67 metabolism and its contribution to plant growth, development and stress adaptation [16,17,22,24-26]. However,  
68 the discovery that a family of plant anion channels, the Aluminium (Al<sup>3+</sup>)-activated Malate Transporters  
69 (ALMTs), are regulated by GABA, and this regulation can modulate tissue growth [18] warrants a re-examination  
70 of the roles of GABA in plants. In particular, this regulation has been proposed to transduce GABA metabolism  
71 into membrane signalling via an alteration of anion flux across cell membranes [27]; as such, this discovery opens  
72 novel research avenues for plant and animal biology [28].  
73

74 Despite being an anion channel – like animal GABA<sub>A</sub> receptors – ALMTs were observed to share little  
75 sequence homology with their proposed animal counterparts, except in a 12 amino stretch that has some similarity  
76 to one important motif for GABA binding in rat GABA<sub>A</sub> receptors [18]. Whilst GABA activates GABA<sub>A</sub> channel  
77 activity in mammals [13], GABA inhibits ALMT activity in plants [18]. However, as the equilibrium potential  
78 for chloride is generally positive in plants and negative in mature animal neurons, GABA inhibition and activation  
79 leads to a relative hyperpolarised state in plants and mature neurons respectively[18,28]. Changes in membrane  
80 potential are a key cellular signal so the finding that GABA alters this in plants, and that this is a prerequisite for  
81 changes in tissue growth [18], suggests that GABA can act as a signal in plants. The fact that GABA can be  
82 present in large concentrations and occurs in every part of the plant examined has been used as an argument  
83 against GABA being a signal in plants [29]; for instance, it can be the main amino acid found in tomato fruit  
84 (~11.5 – 20 mM) [30], and during stress it can often exceed the levels of all other amino acids [22]. The same  
85 argument was used against GABA being a signal in animals in the 1950's and 60's, until the receptor proteins  
86 were identified and local gradients of GABA discovered [31,32] we now have similar evidence in plants (Table  
87 1).  
88

89 In this review, we will provide an update on GABA-regulated ion channels in plants and explore their  
90 possible linkage with GABA-mediated physiological processes to provide an insight into the putative roles of  
91 GABA signalling in plant biology. In the first part of this review, we will compare and contrast ALMTs with  
92 animal GABA<sub>A</sub> receptors in terms of their molecular identity, structure, mode of action and signalling roles. The  
93 aim of this section is to ascertain whether there might be commonalities and differences between GABA signal  
94 transduction in both animals and plants. In the second part of this review we focus on the unique effects that  
95 GABA has on plants and we explore the implications of the discovery that GABA regulates ALMT activity for  
96 transducing signals for the regulation of physiological processes, and the potential interactions between GABA  
97 and other signalling molecules.  
98

### 99 1. Plant ALMTs vs. animal GABA<sub>A</sub> receptors



### 1.1. ALMTs are likely to be involved in signalling

ALMT proteins encode voltage dependent anion channels [33,34] and in at least one case a Rapid or Quick activating Anion Channel (R/QUAC-type) [35]. 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 [36,37] and ROS production in response to bacterial pathogens [38]. When anion channels open, anions are released from the cell tending to depolarise the membrane voltage from its normally very negative resting level [39]. ALMTs are activated by some anions when placed on the efflux side of the channel protein [18,34]. Such transactivation is observed *in vivo* for the R-type anion channels of stomatal guard cells, vacuoles and hypocotyls [38]. 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 [40]. A sensing and signalling role for R-type channels has been suggested [35]. Fig. 1 summarises the factors that regulate the R-Type channels and ALMT anion channels.

### 1.2 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 [41].  $GABA_A$  receptors are chloride ( $Cl^-$ ) channels [6], whilst  $GABA_B$  receptors are G-protein coupled receptors that regulate cation transport (e.g.  $K^+$  and  $Ca^{2+}$ ) [42]. The ionotropic  $GABA_A$  receptor family also includes  $GABA_A$ -rho receptors that are only composed of rho ( $\rho$ ) subunits which forms a distinct ligand gated  $Cl^-$  channels and were previously designated as  $GABA_C$  receptors [32]. 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, interfere with ionotropic glutamatergic transmission, and modulate the excitatory to inhibitory developmental switch dependent upon age [43,44].

An early candidate, touted as a receptor for GABA signalling were the plant glutamate receptor-like proteins (GLRs), which have high sequence similarity to animal ionotropic glutamate receptors (iGluRs) [45]. These possess a regulatory domain with structural homology to the animal  $GABA_B$  receptors [46-48]. They are involved in glycine signalling [49] and are thought to play a role in  $Ca^{2+}$  utilisation, stimulate transient changes in  $Ca^{2+}$  levels and signalling as they behave as ligand gated  $Ca^{2+}$  channels [49-52]. Thus, in plant cells if GABA interacts with GLRs, it should cause transient elevations in cytosolic  $Ca^{2+}$  [49,52]; however, in *A. 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 ALMT in all plants, and all those tested by Ramesh et al. (2015), from wheat, barley, grapevine, Arabidopsis and rice were sensitive to low micromolar concentrations of GABA. An ALMT from Arabidopsis carries a rapid-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 appear to be clear candidates to transduce 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-regulated plant growth and development*).

### 1.3 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,

161 except in a 12 amino stretch important for their regulation by GABA [18]. The GABA<sub>A</sub>, nicotinic acetylcholine  
1 162 (nAChR), GABA<sub>A</sub>- $\rho$ , glycine and 5-HT<sub>3</sub> receptors are members of cysteine (Cys) loop ligand gated ion channel  
2 163 superfamily. The structure of GABA<sub>A</sub> receptors in mammals has been well characterised [63-65]. They are  
3 164 members of the pentameric ligand-gated ion channels (pLGICs), which are ubiquitous neurotransmitter receptors  
4 165 in animals and certain prokaryotic homologues, but are completely absent from multicellular plants and fungi  
5 166 [66]. The eukaryotic members share a motif composed of two Cys residues separated by 13 amino acids (aa)  
6 167 residues [66] and the GABA<sub>A</sub> receptors from different animal species are highly conserved. The mammalian  
7 168 GABA<sub>A</sub> receptor heteromer is composed of three subunits  $\alpha$ ,  $\beta$  and  $\gamma$ , which are associated in a defined ratio to  
8 169 form a functional receptor [67,68]. The ligand binding sites are localised at the  $\beta$  (+) and  $\alpha$  (-) interfaces [69,70],  
9 170 with both  $\alpha$  and  $\beta$  subunits being essential for GABA binding, whilst the subunit composition within the receptors  
10 171 is dependent on the brain regions or on species [71]. For instance, 19 different subunit compositions have been  
11 172 identified in humans that determine the differential GABA binding affinities of GABA<sub>A</sub> receptors and these  
12 173 properties suggest that they can translate diverse GABA signals shaped by development into a functional response  
13 174 [72]. The subunit heterogeneity of GABA<sub>A</sub> receptors determines to some extent whether it mediates phasic  
14 175 (shorter-lasting inhibition typically generated by the activation of GABA<sub>A</sub> receptors following action potentials  
15 176 in a presynaptic interneuron) or tonic (long lasting inhibition generated by GABA conductance activated by  
16 177 GABA in the extracellular synapses) inhibition, as reviewed in [72-76]. The five subunits of GABA<sub>A</sub> receptors  
17 178 form a central pore that remains closed under normal conditions but opens following a conformational change  
18 179 induced by GABA binding [77,78]. Typically a mature subunit is ~450 aa in length, has a hydrophilic extracellular  
19 180 N terminal domain that contains the Cys loop which is the site of action for various drugs, followed by 4  
20 181 hydrophobic transmembrane domains (M1 to M4) and a short C terminal domain. A role for two extracellular Cys  
21 182 residues in agonist binding to the receptor had been suggested [66] but the subsequent mutational studies in  
22 183 GABA<sub>A</sub>, nAChR and glycine receptors suggest otherwise [79-81]. From the solution of the crystal structure of  
23 184 GABA<sub>A</sub>R, the human  $\beta$ 3 homopentamer, details of the ligand binding pocket and key residues in the interaction  
24 185 with agonist are known and these support previous studies identifying key residues in ligand binding for nAChR  
25 186 [82]. The transmembrane domain M2 lines the channel pore and between M3 and M4 is a long intracellular loop  
26 187 that is involved in modulation of the receptor by phosphorylation, protein-protein interactions and post-  
27 188 translational modifications [83,84] (Fig. 2a). A number of proteins that are involved in receptor trafficking and  
28 189 anchoring of receptors to the cytoskeleton and post synaptic membrane interact with the intracellular loop [85,86].  
29 190 It is clear that separate regions on the extracellular domains of the N-terminus form the binding pocket including  
30 191 regions on adjacent subunits. The GABA<sub>A</sub>R  $\beta$ 3 homopentamer comprises regions in a principal face (loops A-C)  
31 192 and a complementary face on an adjacent subunit comprising regions of loops D (Tyr<sup>62</sup>-Gln<sup>64</sup>) and E (Leu<sup>125</sup>-  
32 193 Arg<sup>129</sup>). It is the region of loop D (also referred to as  $\beta$ 2 strand) that was found to show some similarity to a 12  
33 194 residue “motif” in plant ALMTs and a critical phenylalanine that when mutated to cysteine virtually abolished  
34 195 GABA sensitivity [18,87].  
35 196

36 197 In comparison to animal GABA receptors, the structure of ALMTs is poorly understood. It is not known  
37 198 whether the channels are monomeric or can form multimers consisting of homomeric or heteromeric combinations  
38 199 – although we are aware that this is an active area of research. The ALMT genes form a functional protein when  
39 200 expressed alone in *Xenopus laevis* oocytes but whether the channel is formed from multiple subunits or whether  
40 201 a functional GABA binding site can occur in a monomer is not clear. The region of similarity between rat GABA<sub>A</sub>  
41 202 receptor and TaALMT1 is localised at the N-terminus of the former and the C-terminus of the latter. Two studies  
42 203 have predicted the putative TaALMT1 topology [58,88], but the models differ; one suggests that TaALMT1 has  
43 204 6 transmembrane domains (TMDs) and its N and C terminus both face the extracellular space [88], whereas the  
44 205 other, based on phylogenetic analysis of ALMTs across the plant kingdom, predicts that TaALMT1 possesses 8  
45 206 TMDs and its N and C termini are localised to the inside and the outside of cells respectively [58] (Fig. 2c and  
46 207 d). The evidence from the rapid inhibition of malate efflux in *X. laevis* oocytes expressing TaALMT1 by external  
47 208 GABA suggests that the interacting site is localised at the extracellular side or at least rapidly accessible to an  
48 209 intracellular or transmembrane site [18]. Interestingly, based on YFP-QUAC1 (rapid-type anion channels eg.  
49 210 AtALMT12) fusion studies, Mumm et al., (2013) predict that both the N and C termini are located in the cytosol  
50 211 [89]. In silico analysis of Arabidopsis ALMT9 located on the vacuolar membrane, predicts 6 transmembrane  
51 212 domains (TMD) with N terminus facing the cytoplasm [90]. A predicted soluble C terminal domain encompasses  
52 213 nearly half of the protein. Patch clamp analysis of amino acid mutations in AtALMT9 revealed that individual  
53 214 residues affected the function of the channel differently. The removal of positive and negative charges (Lys<sup>93</sup>,  
54 215 Lys<sup>187</sup>, Arg<sup>143</sup>, Arg<sup>226</sup>, Glu<sup>130</sup>) abolished conductivity. Mutation of Arg<sup>200</sup> and Arg<sup>215</sup> affected channel function  
55 216 depending on which residue was substituted, mutation of these residues to asparagine resulted in time dependent  
56 217 inward currents comparable to WT currents whereas mutation to Glu (E) resulted in loss of channel function.  
57 218 Further the sensitivity of point mutations of AtALMT9 to open channel blocker citrate suggested that Lys<sup>193</sup> and  
58 219 Arg<sup>200</sup>, which are located near or within TMa5 are part of the ion conduction pathway of AtALMT9 [90].  
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220 Functional analysis of site directed mutant K193E (insensitive to citrate block) by patch clamping, and ALMT9-  
221 GFP studies, suggested that ALMT9 functioned as a multimer composed of 4 subunits [90].

222  
223 Amino acid residues important for GABA binding in the GABA<sub>A</sub> receptors were identified by  
224 photoaffinity labelling of an affinity purified bovine receptor with [<sup>3</sup>H] muscimol and microsequencing of a  
225 purified labelled peptide [91]. Mutational analysis has identified the key residues essential for GABA binding to  
226 the  $\alpha$ 1 subunit of rat GABA<sub>A</sub> receptors through a point mutation of Phe<sup>64</sup> [92] (Fig. 3). This mutation reduced the  
227 affinities of both agonist and antagonists of rat GABA<sub>A</sub> [92]. Further it has been observed that a similar mutation  
228 in  $\alpha$ 5 subunit had the same effect suggesting that there is close functional and structural association of  $\alpha$ -subunits  
229 with binding sites [92]. Substituted Cysteine Accessibility Method (SCAM) analysis of the amino acids in the  
230 proposed binding region ( $\alpha$ 1 Tyr<sup>59</sup>– Lys<sup>70</sup>) mutated to Cys and expressed with wild type  $\beta$  subunits in HEK293  
231 cells confirmed that F<sup>64</sup>, R<sup>66</sup> and S<sup>68</sup> residues line part of the binding site and that Phe<sup>64</sup> ( $\alpha$ 1F<sup>64</sup>) was very important  
232 in GABA binding [87]. Similar studies in the  $\beta$ 2 subunit confirmed that Tyr<sup>97</sup> and Leu<sup>99</sup> line the GABA binding  
233 site [87]. However, Szczot et al., (2014) have shown that rapid application of agonists to rat recombinant  $\alpha$ 1 $\beta$ 2 $\gamma$ 2  
234 receptors with the  $\alpha$ 1F<sup>64</sup> mutations affected gating, abolished rapid desensitization, slowed current onset and  
235 accelerated deactivation [93]. Further  $\alpha$ 1F64C mutation resulted in a decrease in open channel probability without  
236 affecting channel conductance.

237  
238 Similarly, in plants, site directed mutagenesis has been performed to probe a putative GABA binding site.  
239 In *TaALMT1* mutagenesis of Phe<sup>213</sup> (F<sup>213</sup>) residue appears to affect affinity of GABA action increasing the EC<sub>50</sub>  
240 from 3.4  $\mu$ M to 1.8 mM [18] suggesting that this residue might be important for GABA binding. However, it is  
241 yet to be demonstrated that the mutation of F<sup>213</sup> to C in TaALMT1 affects gating or sensitization (Table 2). The  
242 mutation of equivalent aromatic residue Y (Threonine) in *Vitis vinifera* VvALMT9 to C increases the EC<sub>50</sub> from  
243 6.0  $\mu$ M to 380  $\mu$ M. Nevertheless, these mutations do not completely abolish GABA sensitivity of TaALMT1 [18]  
244 and as such there may be other regions that affect GABA-sensitivity and likely binding of GABA [27]. An *in*  
245 *silico* analysis of 116 different ALMTs revealed that the initial putative GABA-interaction motif appeared highly  
246 conserved across a wide range of plant species [18]. A protein-protein BLAST search of Arabidopsis proteins  
247 using a consensus sequences formed between the GABA<sub>A</sub> and ALMT regions of similarity [18] identified the  
248 majority ALMT members in Arabidopsis as well as other proteins, such as putative F-box protein, ACT-like  
249 protein tyrosine kinases- and an uncharacterised protein (Table 3a and b). However, we do not know if all or any  
250 of these identified proteins are targeted to cell membranes or catalyse ion transport [94,95], therefore if they do  
251 bind GABA they may act through a novel mechanism. It is also possible that the consensus motif alone may not  
252 be sufficient to confer protein GABA-binding ability, and other important regions in ALMTs are also essential.

253  
254 Although no tertiary structure for ALMTs has been resolved experimentally, there are bioinformatics  
255 techniques that can predict this and potential ligand binding sites in a protein. One technique involves examining  
256 homologous protein sequences across a wide range of organisms, and provided there are enough sequences, it is  
257 possible to examine the co-evolution of amino acid residues in a protein [96]. If there is evolutionary coupling  
258 between residues it would imply that they are linked structurally and that they are located near to each other in  
259 the tertiary structure [97]. This can be then used to predict folding in the protein. This technique known as  
260 evolutionary coupling analysis has been used on several proteins to provide structure predictions that turn out to  
261 be very close to known structures from X-ray crystallography, including those for complex ligand activated ion  
262 channels [97]. In the context of ALMTs there are now thousands of homologous protein sequences in the data  
263 bases and these can be harvested to examine evolutionary coupling between residues and to provide insight into  
264 residues in TaALMT1 that may be involved in GABA binding. Submitting the TaALMT1 sequence to the Web  
265 portal EVFold provides data on the coupling between residues over evolutionary time (utilizing 3688 sequences)  
266 and identifies “hotspots” in the protein’s evolution indicating important functional sites [97,98]. Interestingly  
267 residues in the putative GABA motif including F<sup>213</sup> show significant evolutionary coupling (in the top 50 for the  
268 protein) with residues in the N terminus (Fig. 4). These are potential residues involved in forming a GABA binding  
269 pocket [96]. Using these couplings and other information about likely secondary structure and transmembrane  
270 domains, the EVFold computation also predicts tertiary models of the protein of interest. The top-ranking model  
271 is shown in Fig. 4 and displays some of the evolutionarily coupled residues and their proximity to F<sup>213</sup>. In this  
272 region the model predicts that the aromatic side chain is exposed and can form a cavity in the protein, which is  
273 tempting to speculate may accommodate a GABA molecule. Two residues R<sup>40</sup> and Y<sup>96</sup> (among a total of 7  
274 residues) at the N-terminus and start of 1<sup>st</sup> TM showed significant evolutionary coupling with F<sup>213</sup> in the GABA  
275 interaction motif. This information provides the basis to test the model by site directed mutagenesis, particularly  
276 of the residues identified as being closing coupled.

#### 277 278 **1.4 Trafficking, movement and endocytosis**

280 The regulation of the GABA<sub>A</sub> receptor regulation in animals, depends on the number of receptors at the post  
1 281 synaptic membrane either via expression, lateral movement, endocytosis or rate of re-insertion of the receptors  
2 282 into the membrane. Numerous studies have been carried out to understand these processes in glycine and AMPA  
3 283 receptors but relatively little has been published in this regard about GABA<sub>A</sub> receptors [99,100]. However, it has  
4 284 been shown that GABA<sub>A</sub> receptors behave in a similar manner to the glycine and AMPA receptors in that there  
5 285 are both mobile and immobile receptor pools that move laterally in the membrane to regulate the GABA<sub>A</sub> receptor  
6 286 concentrations to adjust to changing environments [101]. In plants, nothing much is known about the trafficking  
7 287 of ALMTs to the plasma membrane or its movements in response to various abiotic or biotic stresses.  
8 288

9 289 Mammalian GABA<sub>A</sub> receptors are constitutively endocytosed and recycled back to the surface of the  
10 290 membrane to regulate the efficacy of the GABAergic transmission [102,103]. Briefly GABA<sub>A</sub> receptors undergo  
11 291 endocytosis via clathrin coated pits [104] by binding of the  $\beta$  and  $\gamma$  subunits to the clathrin adaptor AP2 [105] and  
12 292 require a di-leucine motif for efficient endocytosis [105,106]. Further the expression of the GABA<sub>A</sub> receptors  
13 293 might be downregulated by exposure to GABA and benzodiazepine agonists [107,108]. Preliminary data in plants  
14 294 suggest that GABA mediated inhibition of anion flux is not regulated by endocytosis [18] but more extensive  
15 295 research is needed to understand how the plant receptor is regulated and the role of GABA regulation in plant  
16 296 processes and signalling.  
17 297

### 18 298 **1.5 Pharmacological comparison of ALMTs with GABA receptors**

19 299

20 300 Numerous plant-derived and synthetic pharmacological agents have been used to characterise animal GABA  
21 301 receptors and their role in signalling (either as agonists or as antagonists) [109,110]. These include muscimol,  
22 302 bicuculline, vigabatrin and 3-mercaptopropionic acid (3-MPA) [111,112]. Muscimol (as an agonist) and  
23 303 bicuculline (as an antagonist) are commonly applied to mammalian GABA<sub>A</sub> receptors expressed in heterologous  
24 304 systems to mimic and block GABA action, respectively via their interaction with the GABA binding site [113-  
25 305 115]. Similar effects of both drugs have also been observed on ALMTs expressed in *X. laevis* oocytes. Muscimol,  
26 306 like GABA, reduces TaALMT1-mediated malate efflux but the application of bicuculline abolishes the GABA-  
27 307 inhibited anion flux [18]. A list of other common antagonists/agonists of GABA<sub>A</sub> receptors and GABA-shunt  
28 308 modulators is summarised in Table 4, such as picrotoxin [116], benzodiazepines [117] and flumazenil [118]. Most  
29 309 of the agents listed in Table 4 are of plant origin and have not yet been tested on ALMTs or in plants. GABA  
30 310 mediated regulation is seen in animals, fungi and plants and since many of the agents listed in Table 4 have been  
31 311 used in the characterisation of animal GABA<sub>A</sub> receptors, it would be instructive to test these in plants in regard to  
32 312 their mode of action on GABA mediated regulation of anion channels and signalling in plants. If they also interact  
33 313 with the GABA binding region in ALMTs then it appears that they also have a biological function in plants, it is  
34 314 tempting to speculate that this has been recruited by the medical industry to act on equivalent sites in humans.  
35 315 The alternative hypothesis about the origin of these compounds is that they are synthesised by non-animal systems  
36 316 to act as defence or beneficial compounds. For instance, muscimol, derived from the mycorrhizal fungi *Amanita*  
37 317 *muscaria*, can act as an insecticide by overloading the nervous system of insects. The decaying insects can then  
38 318 be used as a nutrient source for further fungal growth [119].  
39 319

### 40 320 **1.6 Link between aluminium, GABA and calcium in animals and plants**

41 321

42 322 It is perhaps a fascinating coincidence that in both animals and plants, there is interplay between Al<sup>3+</sup>, Ca<sup>2+</sup> and  
43 323 GABA on certain transport proteins and that this has consequences for the development and growth of the  
44 324 organism. Al<sup>3+</sup> is one of the most abundant metals on earth and found in most tissues, but is without an attributed  
45 325 beneficial physiological function [120]. In fact, Al<sup>3+</sup> is associated with toxicity in both animals [121,122] and  
46 326 plants [123-125]. In animals, accumulation within tissues causes various cognitive as well as physiological  
47 327 impairments [126-129] and in plants exposure to Al<sup>3+</sup> causes inhibition of root growth, cytotoxicity and decrease  
48 328 in yield on acidic soils [130-132]. Furthermore, in plants, Al<sup>3+</sup> can inhibit some voltage gated channels and  
49 329 glutamate receptor-mediated currents [133,134]. In humans, Al<sup>3+</sup> toxicity leads to conditions such as dementia,  
50 330 Alzheimers and Parkinsons [135,136]. Aluminium has been shown to potentiate currents evoked by GABA in rat  
51 331 olfactory bulb mitral/tufted neurons [129] but had no effect on membrane currents induced by glutamate, glycine,  
52 332 N-methyl-D-aspartate or kainate. It has been suggested that the GABA<sub>A</sub> receptors express two allosteric sites for  
53 333 Al<sup>3+</sup>: one a high affinity binding site (potentiating) and the other a low affinity binding site (inhibiting) and the  
54 334 effect of Al<sup>3+</sup> further depends on the subunit composition of the receptors. In adult male albino rats either fed with  
55 335 Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> in different doses or untreated, the levels of glutamate and glutamine increased in a dose dependent  
56 336 manner in the brain tissue, while the GABA levels decreased [137] compared to controls. The mechanisms by  
57 337 which Al<sup>3+</sup> causes changes in glutamate, glutamine or GABA levels in brain is not very clear and one hypothesis  
58 338 is that Al<sup>3+</sup> may induce modifications in the enzymes of the GABA shunt leading to neurotoxicity and  
59 339 neuropathology.  
60 339

340

1 341 In plants it is well known that  $Al^{3+}$  causes rhizotoxicity, impairs root growth and overall yield of plants  
2 342 in acidic soils [124,138]. TaALMT1 confers  $Al^{3+}$  tolerance in wheat roots through  $Al^{3+}$  ion activating TaALMT1  
3 343 causing the release of malate that complexes the external  $Al^{3+}$  [139]. GABA inhibition of TaALMT1 is thought  
4 344 to modulate the malate efflux and to provide a link between malate production and malate release under  $Al^{3+}$  stress  
5 345 [27]. GABA is synthesised in the cytoplasm and enters mitochondria via GABA permease [140] but inhibition of  
6 346 malate efflux suggests that GABA signalling occurs in the apoplast. The question then arises as to how GABA  
7 347 enters the apoplast and exits the cell. GABA is taken up into the cells via the high affinity GABA uptake  
8 348 transporter GAT1 [141] and is then perhaps regulated by signalling in the cell via regulation of GAT1 and other  
9 349 GABA transporter/s. Interestingly no GABA efflux transporter has been identified to date. Unlike animal systems  
10 350 [142], there is little information or experimental evidence on  $Ca^{2+}$  regulation of GAT1 from *Arabidopsis*. The  
11 351 expression of 7 of the 9, 14-3-3 genes identified in *Arabidopsis* seedlings is down regulated by GABA (10 mM)  
12 352 in the presence of high  $Ca^{2+}$  (22 mM) and requires functional ethylene and ABA signalling pathways [53], while  
13 353 low  $Ca^{2+}$  (2 mM) did not affect the transcripts. It would be interesting to study the expression of GABA shunt  
14 354 genes and ALMTs in root tips in presence and absence of different concentrations of  $Ca^{2+}$ ,  $Al^{3+}$  and exogenous  
15 355 GABA to understand if there is an interaction between all three similar to animals.

### 16 356 **1.7 Evolutionary insights into ALMTs and GABA<sub>A</sub> receptors**

17 357  
18 358  
19 359 Gene and genome duplication has been documented as one of the most important factors in the evolution of  
20 360 eukaryotic animals and plants [143-145]. Gene duplication followed by gene divergence is thought to be the  
21 361 underlying factor in evolution of central nervous system in vertebrates [146]. Both the cationic (acetylcholine,  
22 362 serotonin) and anionic (eg. GABA, glycine) ligand gated channels have been predicted to have diverged before  
23 363 the origin of eukaryotes [147]. Despite this plants do not possess any orthologous proteins to the mammalian  
24 364 GABA receptors, suggesting that ALMTs may have evolved convergently to fulfil a GABA-signalling role.  
25 365 GABA<sub>A</sub> receptors are made up of multiple subunits and fourteen of the human GABA<sub>A</sub> receptor genes cluster on  
26 366 four chromosomes [148,149]. Two clusters contain 2 genes encoding  $\alpha$ , one gene encoding  $\beta$  and  $\gamma$  subunits each  
27 367 while the other two clusters contain genes encoding  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\epsilon$  subunits [148]. Evidence suggests that the four  
28 368 clusters arose from the duplications of and within a single GABA<sub>A</sub> receptor gene cluster with  $\alpha$ ,  $\beta$  and  $\gamma$  subunits  
29 369 encoded for by single genes [148,149]. It is thought that  $\epsilon$  and  $\pi$  subunits also arose from gene duplications but  
30 370 not from the same four clusters [149]. Further, an ancestral GABA<sub>A</sub> receptor gave rise to two monophyletic clades:  
31 371 one that has subunits that are involved in binding to benzodiazepines ( $\alpha$ ,  $\epsilon$  and  $\gamma$ ) and the other that is not involved  
32 372 in binding to benzodiazepines ( $\rho$ ,  $\beta$ ,  $\Delta$ ,  $\theta$  and  $\pi$ ) and this divergence occurred before the split from urochordates  
33 373 [150].  
34 374

35 375 Whole genome duplications are thought to be the main source of gene duplications in plants, although  
36 376 individual gene duplications are also common [151]. It is thought that numerous genome duplication events have  
37 377 occurred during the diversification of angiosperms including polyploidy [152-155]. Phylogenetic analyses of  
38 378 ALMT proteins from plants such as *A. thaliana*, *P. trichocarpa*, *O. sativa*, *S. moellendorffii* and moss *P. patens*  
39 379 subdivided these proteins into five distinct clades [39]. The ALMT family was initially thought to be specific to  
40 380 angiosperms but now it has been shown that ALMTs are present in Bryophyta (mosses) and Lycopphyta [58] and  
41 381 possibly algae [156]. Interestingly, no ALMTs have so far been identified in bacteria, fungi, humans, or amoeba,  
42 382 though the ALMTs share a domain of similarity to the fusaric acid resistance protein (FusC) effluxers in bacteria  
43 383 (REF). Phylogenetic analyses of 400 non-redundant ALMT proteins identified from 30 embryophyte species and  
44 384 2 chlorophytes revealed that all belonged to a single group of orthologs indicating that they arose from a single  
45 385 ALMT type protein [58]. However, it was observed that ALMT proteins from *S. moellendorffii* and *P. patens*  
46 386 formed two distinct groups in addition to five clades identified [39,58]. Furthermore the different clades/groups  
47 387 arose by several gene duplication events in different lineages and underwent functional diversification e.g.  
48 388 ALMTs from *Arabidopsis* [39]. When an *in silico* analysis of 116 ALMTs was carried out for the GABA motif  
49 389 from ALMTs from plants, it was observed that there were natural variants (Cys for Phe) in the amino acid residue/s  
50 390 that appear to be important for GABA binding [18]. This would potentially render such variants insensitive to  
51 391 GABA, but so far these have not been examined. Given the structural and functional diversity of full length ALMT  
52 392 proteins, we performed a phylogeny of the amino acid motif important for GABA binding from the ALMTs used  
53 393 by Dreyer et al., (2012) in their phylogenetic analyses and also wheat (*T. aestivum*), barley (*Hordeum vulgare*)  
54 394 and rice (*Oryza sativa*) (Fig. 5) [58]. The motif for GABA<sub>A</sub> receptor from rat was used as an outgroup. It is  
55 395 interesting to note that the motif region from different ALMTs fall into similar clades identified for the full length  
56 396 proteins [58]. The motif region from TaALMT1 from wheat, HvALMT1 from barley and OsALMT5 from rice  
57 397 fall into the evolutionary clade 1 with ALMTs 1, 2, 7 and 8 from *A. thaliana*. It is interesting that ALMTs from  
58 398 wheat (TaALMT1), barley (HvALMT1), rice (OsALMT5) and *Arabidopsis* (AtALMT1) have been shown to be  
59 399 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 (and has not been reported) [18]. Based on consensus sequence, sequence logo and residues in the 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 plants that can be predicted to be GABA sensitive. Interestingly clade 2 in this analysis splits into 2 parts – a and b (in comparison to phylogenetic analysis by Dreyer et al., 2012). Clade 2a has plants that are predicted to be GABA sensitive while clade 2b has plants that may be weakly sensitive to GABA based on the fact that the 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.

## 2. GABA regulation in plants

### 2.1 GABA regulates plant growth and development

The comparison above between plant ALMTs and mammalian GABA<sub>A</sub> receptors indicates that ALMTs may respond to GABA in an analogous manner to that of GABA 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 [157]. 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 [157]. 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 2-3 fold increase in plant growth was observed over that cultured in nutrient solution alone and 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 [157] and soybean hypocotyl tissue [158]. In sunflower, the effect of GABA was dose dependent with low concentrations promoting growth and high concentrations inhibiting growth [159]. 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 [19,160,161]. Exposure to 10 mM GABA, further increased tissue GABA concentrations in a *GABA-T* T-DNA insertion line (named *gaba-t* or *pop2*) [19,161]. 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 [162]. 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* [163-165], nodule formation in *Medicago* [166-168] and control of leaf senescence in *Arabidopsis* [169]. Endogenous GABA concentrations exhibit a light-rhythm dependent oscillation in *Arabidopsis* tissue [170], suggesting 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 Section 2.8 below).

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 [19,161]. GABA regulation of pollen tubes is widespread across the plant kingdom with effects observed for both angiosperms and gymnosperms [18,171]. Pollen germination and polarization of *Picea wilsonii* is affected by GABA, supplementation with GABA between 50 to 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 [172,173]. Pollen tube growth of both *Arabidopsis* and grapevine was also found to be inhibited by muscimol (an agonist of GABA<sub>A</sub> 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 [41], 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.  $\text{Ca}^{2+}$ ) and efflux (e.g.  $\text{Cl}^-$ ) across the plasma membrane that drives oscillations in cytosolic ion concentrations [60,172-175]. It was observed that 1  $\mu\text{M}$  GABA increased cytosolic  $\text{Ca}^{2+}$  in *N. tabacum* pollen protoplasts [20], and 1 mM GABA elicited a  $\text{Ca}^{2+}$  influx into pollen tubes through a pathway independent of glutamate-induced increases in cytosolic  $\text{Ca}^{2+}$  (1  $\mu\text{M}$  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  $\text{Ca}^{2+}$  influx, whereas these currents were inhibited by 100 mM GABA [20]. As GAD is activated by increases in cytosolic  $\text{Ca}^{2+}$ , GABA-induced  $\text{Ca}^{2+}$  influx will potentially affect production of GABA and feedback on ion flux across the membrane that may modulate pollen tube growth [20].

## 2.2 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*  $\times$  *canescens*, rice, and soybean [189-195]. However, the molecular mechanism behind the GABA increases and its consequences has only been probed in Arabidopsis [160,177,190,192,193,195,196]. The Arabidopsis seedling produced  $\sim 15 \mu\text{moles.g}^{-1}$  DW level of GABA under 150 mM salt stress in shoots, this was approximately 20-fold higher than in non-stressed conditions ( $0.7 \mu\text{moles.g}^{-1}$  DW) [160]. The Arabidopsis GABA transaminase (*GABA-T*) mutant (*gaba-t* or *pop2*), which blocks GABA catabolism and causes GABA accumulation (see Section 2.9), 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 wildtype [160]. The investigation of global transcriptional profile found that the *pop2* mutant lines had 10 cell-wall related (4 up-regulated and 6 down-regulated), 8 carbon metabolism (up-regulated) and 3 polyamines metabolism genes differentially expressed, consistent with metabolomics analysis showing that central carbon metabolism was disrupted by salt stress [197]. 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 [161]. Thus, it was proposed that GABA-mediated response to salt-stress involves regulation of central carbon metabolism and cell wall modification [160,197]. Intriguingly no *ALMT* gene transcripts were found to be regulated by salt stress in this study.

Drought stress was reported to promote GABA synthesis in Arabidopsis, soybean, sesame (*Sesamum indicum* L.), bean (*Phaseolus vulgaris* L. cv. Topcrop) and turnips (*Brassica rapa* L. var. Shogoin) [181,188,198-200]. 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* $\times$ *pop2-5*, increased endogenous GABA production and rescued the drought sensitive phenotype of *gad1/gad2* and recovered stomatal conductance to wildtype levels [188,201]. So 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. ALMT12) [188].

## 2.3 GABA regulates $\text{Al}^{3+}$ tolerance in plants

A common problem in acidic soils is that  $\text{Al}^{3+}$  becomes soluble in the soil solution. In wheat, two near-isogenic lines (NILs) – ET8 ( $\text{Al}^{3+}$ -tolerant) and ES8 ( $\text{Al}^{3+}$ -sensitive) were first isolated at a single locus designated as *Alt1*, essential for root  $\text{Al}^{3+}$  sensitivity by Delhaize et al. (1993) [202,203]. Later, the gene *TaALMT1* was identified as underpinning the locus *Alt1* as the protein that facilitates malate efflux from root tips, which chelates  $\text{Al}^{3+}$  and prevents  $\text{Al}^{3+}$ -inhibition of root growth. The high expression of *TaALMT1* in ET8 compared to ES8 is believed to confer the difference in  $\text{Al}^{3+}$  sensitivity between the two NILs [204]. Interestingly, the  $\text{Al}^{3+}$  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  $\text{Al}^{3+}$  was present, GABA concentrations were lower in the root tips of ET8 compared to when  $\text{Al}^{3+}$  was absent, and this coincided with the induction of malate efflux [18]. Treatment with GABA inhibited malate efflux under these conditions and abolished  $\text{Al}^{3+}$  tolerance in roots [18]. The down regulation of GABA is essential for plant adaptation to acidic ( $\text{Al}^{3+}$ ) stress. This led to the discovery that *TaALMT1*, and other ALMTs more broadly can have their transport activity regulated by GABA [18]. Notably, GABA ( $\sim 2 \mu\text{M}$ ) was previously found as one predominant molecule

519 in root exudates (followed by putrescine, alanine, betaine and glutamate) at near neutral pH (6.5-6.8) [205]. It has  
1 520 been suggested that wheat can reuptake a range of organic nitrogen compounds at sub micromolar concentrations  
2 521 from root exudates. A number of transporters have been identified to be involved in secretion of root exudates  
3 522 [206,207] but to date the mechanism of GABA efflux from roots has not been identified.

#### 4 523 5 524 **2.4 GABA regulates plant defence**

6 525  
7 526 GABA rapidly accumulates in the apoplast following herbivory attack and pathogen infection and it is used in  
8 527 defence responses, and possibly signalling [201,208-212]. The rapid increase in GABA by 5-fold in tobacco was  
9 528 detected within 10 min of the leaf being crawled upon by the tobacco budworm (*Heliothis virescens*) and by 11-  
10 529 fold in soybean following leaves being crawled upon by *Choristoneura rosaceana* cv Harris larvae [213].  
11 530 Transgenic tobacco plants overexpressing a petunia *GAD* gene achieved a higher tissue GABA concentration and  
12 531 conferred more resistance to *Meloidogyne hapla* than wildtype plants with significantly fewer egg masses on the  
13 532 root surface by  $\geq 50\%$  [209]. The triple mutant *gad1/gad2 $\times$ pop2-5* line had a greater GABA content within tissue  
14 533 and a greater resistance against insect herbivores *S. littoralis* than wildtype Arabidopsis [201]. These observations  
15 534 point to a positive correlation between GABA induction and herbivory defence [210]. This GABA increase is  
16 535 considered to cause physiological disorders to insect larvae via the inhibition of their neuronal GABA-targeted  
17 536 Cl<sup>-</sup> channels that results in a reduced growth and survival rate [210,214-218].  
18 537

19 538 In plant-microbial interactions, GABA is also induced and has a positive contribution to plant defence  
20 539 against microbial invasion. The application of cell-wall elicitor derived from rice blast fungus (*Magnaporthe*  
21 540 *grisea*) remarkably increased GABA content by 12.5-fold in rice suspension cultured cells [211]. Exogenous  
22 541 application of GABA enhanced the resistance of tomato to *Botrytis cinerea* [219]. To further explore the GABA  
23 542 correlation with pathogen defence, Park and co-workers (2010) deleted 3 GABA transaminase genes (*GabT*) in  
24 543 *Pseudomonas syringae* DC3000 to generate a triple mutant strain *-ΔgabT2/T3/T1* with a defect in GABA  
25 544 degradation activity resulting in approximately 2.5-fold higher levels of GABA than in wildtype. This mutant *P.*  
26 545 *syringae* strain *ΔgabT2/T3/T1* weakened its infection on Arabidopsis leaves, and following a disruption of *GABA-*  
27 546 *T* in *pop2* mutants from Arabidopsis, *ΔgabT2/T3/T1* displayed further reduced colonization [212]. This advocates  
28 547 that pathogen induced GABA production by plants, on the one hand, is positively correlated with its microbial  
29 548 resistance, while on the other hand, the ability of a pathogen to metabolize GABA is associated with their infection  
30 549 capacity. The mechanism behind GABA-mediated defence against *P. syringae* is unclear however, we can see  
31 550 some hints from plant interaction with *Agrobacterium tumefaciens* [220-222]. *A. tumefaciens* produces crown  
32 551 galls on infection, and the level of quorum-sensing signal [N-(3-oxooctanoyl) homoserine lactone-OC8HSL] was  
33 552 inactivated by GABA [220]. Two GABA-binding proteins have been identified from *A. tumefaciens* –the non-  
34 553 selective GABA sensor Atu2422 (binding to a broad spectrum of amino acids) and the selective GABA sensor  
35 554 Atu4243, both of which are critical for the inactivation of OC8HSL quorum-sensing signal [222,223]. An analysis  
36 555 of Atu4243 crystal structure identified serial conserved residues for GABA interaction  
37 556 (W<sup>8</sup>T<sup>12</sup>E<sup>60</sup>F<sup>99</sup>Y<sup>101</sup>W<sup>200</sup>R<sup>203</sup>D<sup>226</sup>Y<sup>262</sup>), which is also possessed by *P. syringae* (W<sup>8</sup>T<sup>12</sup>E<sup>60</sup>F<sup>99</sup>F<sup>101</sup>W<sup>200</sup>R<sup>203</sup>D<sup>226</sup>Y<sup>262</sup>)  
38 557 [222], implicating that plants may have similar machinery for GABA-mediated defence against both *A.*  
39 558 *tumefaciens* and *P. syringae*. Intriguingly, these key GABA-interaction residues from Atu4243 do not appear in  
40 559 the plant or animal GABA-regulated region (as reviewed in Section 1.3). So far, however, no evidence is available  
41 560 to indicate any GABA-regulated ion flux or channel involved in this plant-microbial interaction.  
42 561

#### 43 562 **2.5 Crosstalk between GABA and other signalling molecules / hormones**

44 563  
45 564 GABA has been proposed to be a stress-related metabolite with links to plant hormones [22,224-227] and the  
46 565 oxidative burst [180,183,228-230]. Exogenous GABA has been reported to promote ethylene synthesis in  
47 566 sunflower and *Stellaria longipes* [159,231]; however, it reduced ethylene production in *Caragana intermedia*  
48 567 roots under salt stress [232]. Alternatively, perturbed ethylene levels also impairs GABA metabolism in plants.  
49 568 The exogenous application of ethylene inhibitor (aminoethoxyvinylglycine, AVG) decreased GABA  
50 569 accumulation in Creeping bentgrass (*Agostis stolonifera*) (cv. Penncross) under heat stress [227]. The ethylene  
51 570 inhibitors AVG and AIB (amino isobutyric acid) promoted Al<sup>3+</sup>-activated malate efflux from the root tips of wheat  
52 571 ET8 line [233], while ethylene donor (Ethrel) inhibited Al<sup>3+</sup> induced efflux from tobacco cells when expressing  
53 572 *TaALMT1* [233]. Coupling with the evidence that Al<sup>3+</sup> stress reduces endogenous GABA production leading to  
54 573 increased malate efflux [18] (as discussed above), we speculate that the application of ethylene inhibitor somehow  
55 574 modulates GABA concentrations or perhaps ALMT expression to maximise malate efflux [18,233]. There may  
56 575 also be a cross talk between GABA and ethylene that confers a negative regulation of malate efflux, perhaps via  
57 576 regulation of TaALMT1 activity.  
58 577



578 Other hormones can also affect GABA metabolism in plants. A T-DNA insertion into the *NCED3* (*9-*  
579 *cis-epoxycarotenoiddioxygenase 3*) gene in *Arabidopsis* impaired dehydration-induced abscisic acid (ABA)  
580 synthesis [225,234,235] and the mutant had a significantly higher GABA accumulation compared to wildtype  
581 [225]. An overexpression of two DELLA subfamily members—*gibberellins (GAs) insensitive* gene (*GAI*) and  
582 repressor of *GAI*-like (*RGL1*) in *Populus* seedlings increased GAs level by 12 and 64-fold respectively; while  
583 GABA was also 3-fold higher in these transgenic seedlings compared to wildtype [236]. Chilling treatments were  
584 found to increase GABA content in loquat fruit and this GABA was further increased when methyl jasmonate  
585 (MeJA) was applied in addition to chilling [237]. However, its role in plant-herbivory interaction was not tested,  
586 although both GABA and MeJA appear to contribute to plant defence against herbivory attack [201,238]. In  
587 *Arabidopsis*, the triple T-DNA insertional mutant of *GAD1*, *GAD2* and *GABA-T* (*gad1/gad2×pop2* line) over  
588 accumulated GABA and displayed better systemic defence against the insect herbivore *Spodoptera littoralis*  
589 [201], whereas the levels of defence hormone against *S. littoralis* –jasmonate (JA) and its bioactive derivative,  
590 (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) showed no obvious difference [201,239]. GABA may thus interplay  
591 with plant hormones, such as ABA, GAs and JA, but possibly only upon certain stimuli (e.g. JA). Taken together,  
592 an interaction between GABA metabolism and hormone production is likely to modulate several physiological  
593 processes in plants and requires further research.

594  
595 Apart from hormones, GABA metabolism has also been shown to have links with ROS production (e.g.  
596 H<sub>2</sub>O<sub>2</sub>). When *C. intermedia* was grown in 300 mM NaCl, endogenous H<sub>2</sub>O<sub>2</sub> gradually increased in root and shoot  
597 tissue for up to 72 h [232]. However this was abolished by exogenous application of GABA [232]. Similarly,  
598 potassium cyanide treatment, which mimics hypoxia stress, stimulated H<sub>2</sub>O<sub>2</sub> production in grapevine buds, and  
599 again this was reduced by GABA [186]. Elicitors from rice blast fungus (*Magnaporthe grisea*) increased GABA  
600 production and decreased GABA-T activity, and the activation of ROS scavenging recovered the GABA-T  
601 activity in this case [211]. Thus, GABA was proposed to protect plants from oxidative stresses [16,208].

## 602 603 **2.6 Cytosolic pH modulates ALMT activity**

604  
605 A model has been recently proposed by Gilliham and Tyerman (2015) for regulation of plasma membrane ALMT  
606 activity by malate and GABA, which respectively contributes to a positive and a negative regulation of TaALMT1  
607 activity. This confers a connection of metabolism with membrane signal sensing [27]. On the one hand, malate is  
608 a metabolite regulated by cytosolic pH: 1) high cytosolic pH increases malate synthesis as it consumes OH<sup>-</sup>; 2)  
609 low cytosolic pH inhibits malate synthesis and stimulates its metabolism into pyruvate together with CO<sub>2</sub> and OH<sup>-</sup>  
610 release [240]. On the other hand, GABA is also regulated by cytosolic pH [16]: i) the acidic pH stimulates the  
611 synthesis of GABA (via up regulation of GAD activity); ii) this process is reversible when increasing cytosolic  
612 pH [241,242]. Taken together, it appears that high cytosolic pH stimulates malate production and suppresses  
613 GABA leading to a relatively low GABA-to-malate ratio, and likely a high ALMT activity. Whereas cytosolic  
614 acidification will shift to a higher GABA-to-malate ratio that reduces activity of plasma membrane ALMTs.  
615 Therefore, changes in cytosolic pH induced by stresses (e.g. salt and hypoxia) possibly alters ALMT activity, and  
616 then changes in cell membrane voltage and transport to elicit downstream response [243,244].

## 617 618 **2.7 The GABA-malate connection at the tonoplast**

619  
620 The model proposed by Gilliham and Tyerman (2016) as described in the section above connects the GABA-  
621 malate metabolism to the plasma membrane signal mediated by ALMTs. In fact a number of ALMT family  
622 members (e.g. AtALMT6 and VvALMT9) are also targeted to the tonoplast membrane [56,57,61,245]. For  
623 instance, *ALMT9* from grapevine encodes a vacuolar membrane malate channel sensitive to GABA at high affinity  
624 (6 μM) when expressed in *X. laevis* oocytes [18,61]. Presumably the model proposed by Gilliham and Tyerman  
625 (2015) on plasma membrane regarding the ALMT-mediated GABA-malate signalling paradigm could be mirrored  
626 at the tonoplast [27]. Thus stress-induced GABA elevation in the cytoplasm could transiently increase the  
627 cytosolic GABA:malate ratio to negatively modulate tonoplast ALMT activity and reduce malate uptake from  
628 cytoplasm into vacuoles. This will also lead to a change in vacuolar membrane potential and perhaps other ion  
629 fluxes across the tonoplast. The tonoplast localized GABA transporters, such as the cationic amino acid  
630 transporters (CATs) from *Solanum lycopersicum* (SICAT9) catalyses GABA uptake into vacuoles [246], and may  
631 have a similar role to that of GAT1 in this model.

## 632 633 **2.8 Is GABA involved in long-distance transport regulation?**

634  
635 A range of signalling molecules can be translocated between shoot and root via the plant vascular system,  
636 including hormones, ROS and salicylic acid (SA), as reviewed in [247,248]. GABA has been found in the xylem  
637 sap of walnut [249] and salt treatment increases GABA in the root xylem of soybean [250]. Approximately 0.7

638  $\mu\text{mol g}^{-1}$  GABA was present in soybean nitrogen-fixing nodules however only 0.01  $\mu\text{mol g}^{-1}$  GABA and almost  
1 639 no GAD activity were detected in bacteroids of cowpea *Rhizobium* MNF2030, suggesting that GABA in the  
2 640 nodules was probably supplied by the host instead of *in vitro* synthesis [166]. Artificial feeding of 15 mM GABA  
3 641 to *M. truncatula* petioles doubled GABA concentration in nodules, and enhanced nodule activity and  $\text{N}_2$  fixation  
4 642 [251]. In this case, more GABA was likely transported into nodules and might be correlated with the observed  
5 643 increases in nodule activity and  $\text{N}_2$  fixation. Nevertheless, it is uncertain whether this rapid change of GABA  
6 644 levels in nodules was due to translocation via xylem or phloem from one part of the plant to another, or due to *de*  
7 645 *novo* synthesis in response to stresses (e.g. wound) [252]. So whether GABA is involved in long distance transport  
8 646 within plants still remains inconclusive and hard to probe [252]. The development of a fluorescence GABA sensor  
9 647 and its application to intact plants would be of benefit to such studies [253].

## 11 649 **2.9 Elements that shape GABA signals within in plants via the GABA shunt**

12 650  
13 651 In mammalian neuron cells, a GABA signal is generated via GABA synthesis in presynaptic cells from Glu  
14 652 catalyzed by two GAD enzymes, GAD65 and GAD67 [254]. GABA is then transported via vesicles by a vesicular  
15 653 neurotransmitter transporter (VGAT) [255] and released into the extracellular space for activation of GABA  
16 654 receptors and inhibitory neuron signal transmission. The GABA signal is terminated via re-uptake by surrounding  
17 655 glial cells through plasma membrane GABA transporters (GATs) [256] and degraded by GABA-T [257,258].

18 656  
19 657 The enzymes engaged in the GABA shunt are conserved in both animal and plant kingdoms [13,162].  
20 658 GABA is synthesized from Glu in the cytoplasm by GADs with  $\text{CO}_2$  release in plants [14] and mammals  
21 659 [157,259]. The C-terminus of GAD2 from Arabidopsis and rice contains an autoinhibitory CaM-binding region,  
22 660 the deletion of which increases GAD2 activity by 40 fold in rice and leads to GABA overproduction by 100-fold  
23 661 in seedlings [260]. In Arabidopsis, CaM T-DNA insertion mutant lines *cam1*, *cam4*, *cam5-4*, *cam6-1* and *cam7-*  
24 662 *1* seedlings, there is significantly more GABA produced by  $\text{H}_2\text{O}_2$  and paraquat treatments [183], so  $\text{Ca}^{2+}$ /CaM  
25 663 indirectly regulates GABA metabolism and GABA accumulation in plants [157,162,170,261,262]. GABA is taken  
26 664 up into mitochondria through a mitochondrial-localized GABA permease (GABP) [140] and catabolised by  
27 665 GABA-T into succinic semialdehyde (SSA) and finally succinate [29,252,263] this process is similar to the  
28 666 biological process in mammals [13]. In Arabidopsis, knocking out *GABA-T* (*pop2/gaba-t*) blocks GABA  
29 667 degradation resulting in more than 10-fold GABA over accumulation [19,160,161,201,264]. Succinate  
30 668 semialdehyde (SSA) as the downstream metabolite of GABA is further catabolised into succinate by succinate  
31 669 semialdehyde dehydrogenase (SSADH) [263]. The disruption of this single *SSADH* gene in Arabidopsis causes  
32 670 necrosis, constant higher GABA and  $\text{H}_2\text{O}_2$  over accumulation, and leads to hypersensitive to light and heat stress  
33 671 [24,263,265,266]. In *ssadh* mutant lines, the hypersensitive phenotype is partially relieved by treatment with  
34 672 vigabatrin as an inhibitor of GABA-T and GABA degradation [263,265,266]. Interestingly, crossing *ssadh* with  
35 673 *pop2-4* generates *ssadh/pop2-4* line that has higher GABA levels in tissue, rescues *ssadh* dwarf and hypersensitive  
36 674 phenotypes, and with  $\text{H}_2\text{O}_2$  production at basal levels similar to wildtype seedlings [267]. SSADH is also reported  
37 675 to control the robust leaf patterning and formation of the adaxial–abaxial axis of leaf primordia through a screening  
38 676 of *enlarged fil expression domain1* (*enf1*) mutant (*enf1 = ssadh*) [268]. Vigabatrin has not been applied to test its  
39 677 effect on the *enf1* mutant, but the *enf1/gaba-t (=ssadh/pop2-4)* has a wildtype-like leaf patterning [268]. The  
40 678 manipulation of tissue GABA levels through a T-DNA insertional mutation of Arabidopsis *GABA-T*, *GAD* and  
41 679 *SSADH* can be phenocopied in tomato via virus-induced silencing of their homologs from tomato (*SIGABA-Ts*,  
42 680 *SIGADs* and *SISSADHs*, respectively) [269]. A study led by Seher et al (2013) have measured tissue Glu, GABA,  
43 681 succinate and total nitrogen concentrations as well as glutamate dehydrogenase (GDH) and GAD activities in 16  
44 682 different plant species. They found that a large variation in GAD and GDH activity appears between different  
45 683 plant species and this does not match their endogenous N, Glu and GABA content [270]. Accordingly, the tissue  
46 684 GABA levels are not simply determined by one or two enzymes. It appears that GADs, GABA-T, SSADH and  
47 685 GAD interact, with  $\text{Ca}^{2+}$ /CaMs impacting on GABA production in all cases. Their interaction, perhaps together  
48 686 with other elements, e.g. carbon metabolism via tricarboxylic acid cycle (TCA cycle) and malate [27,62]  
49 687 coordinate the generation and/or termination of GABA signals. The perturbed tissue GABA levels via the  
50 688 manipulation of these GABA shunt elements has successfully impaired GABA-mediated signalling and helped us  
51 689 explore GABA-metabolism and -mediated signalling in plants. Nevertheless, these different elements essentially  
52 690 display differential cell-type expression patterns [19,161,268], thereby certain GABA signals may be shaped only  
53 691 in particular cell types. In this case, a cell-type modification of GABA shunt elements possibly causes a cell-  
54 692 specific GABA signalling perturbation, which is necessary to dissect the GABA roles in different cell types and  
55 693 particular physiological processes.

56 694  
57 695 In addition, the disruption of elements in the GABA shunt is not always associated with perturbed GABA  
58 696 concentrations but yet still alters plant growth, development and stress responses [140,271]. The T-DNA insertion  
59 697 into either *GABP1* or *GAT1* fails to change GABA levels in mutant tissue [140,272]. Knocking out *GABP1*

698 significantly reduces mitochondrial GABA uptake rate by > 40% and lowers CO<sub>2</sub> evolution (approximately 20%)  
699 so that it impairs GABA flow into the TCA cycle and mitochondrial respiration [140]. The high-affinity GABA  
700 transporter, GAT1 localized at the plasma membrane is thought to only reuptake GABA into the  
701 cytoplasm[27,271], reminiscent of mammalian GATs As such supply of exogenous GABA does not increase  
702 tissue GABA level in the *gat1* mutant, the disruption of *GAT1* caused no change in tissue GABA levels but altered  
703 the metabolic carbon-nitrogen equilibrium and response to low carbon - and nitrogen- environment in plants (e.g.  
704 Glu, malate, fructose and etc) [272]. These two cases indicate that the disruption of certain GABA shunt elements  
705 does not always alter GABA concentrations in plants; however, it may still impair GABA-associated  
706 physiological processes.

## 707 708 **Conclusions and Future Research**

709 The recent discovery of plant GABA regulated ion channels – ALMTs, opens up new pathways for GABA  
710 research in plant biology, here our review provides an insight into the similarity and differences between plant  
711 ALMTs and animal GABA<sub>A</sub> receptors, the molecular determinants of GABA regulation by ALMTs proteins, the  
712 connection between GABA metabolism with GABA-mediated ion flux and physiology, and elements shaping  
713 potential GABA signals in plants. The comparison of literature from animals and plants suggests that common  
714 features exists in both such as: i) residues important for GABA sensitivity; ii) GABA regulation of anion flux;  
715 and, iii) common drugs that modulate GABA receptor activity, as well as differences such as; i) limited homology  
716 in predicted full length amino acid sequence of the GABA<sub>A</sub> receptor (similarity is restricted to a 12 amino acid  
717 stretch); ii) topology – mammalian receptor has 4 transmembrane domains while the plant ALMT has 6 (or more)  
718 predicted transmembrane domains; iii) mammalian receptor is heteropentamer while nothing much is known in  
719 plants regarding the subunits but we do know that plant receptor can function as a homomer since the expression  
720 of only one gene is sufficient to elicit functional response to GABA; and, iv) GABA binding site in mammalian  
721 receptor is located at the N terminus while the predicted binding site in ALMT is located at the end of  
722 transmembrane 6. Interestingly, most of the drugs that are modulators of mammalian GABA receptors are of plant  
723 origin and therefore the application of these drugs could well interact with the predicted GABA binding region in  
724 ALMTs and will help further elucidate the molecular identity and basis of GABA regulation of ion fluxes in  
725 plants.

726  
727 The characterisation of the predicted GABA binding motif in plants is still in its infancy and there are  
728 key research gaps. It remains to be shown: i) whether GABA binds to the identified aromatic amino acids residues  
729 in ALMT1; ii) what residues line the binding site and the pore; iii) the kinetics of GABA binding; iv) whether  
730 there are more than one region in the ALMT proteins involved in GABA mediated regulation; v) whether there  
731 are other metabolites such as amino acids and compounds related to GABA metabolism that are involved in  
732 regulation of ALMTs/ion channels; and, vi) what the tertiary structure is of ALMTs. Additionally, a number of  
733 GABA-mediated physiological processes in plants may require the participation of ALMTs to transduce GABA  
734 metabolism into plasma- and/or tonoplast-membrane signalling. The interaction between GABA and other  
735 signalling molecules may also contribute to certain responses albeit the candidates remain elusive. GABA signals  
736 controlled by GABA shunt elements appear to be shaped in particular cell types, although it is still inconclusive  
737 whether GABA signals are involved in long distance translocation within plants. However, the recent research on  
738 plant GABA highlighted in this review suggests that new insights into the GABA regulation of physiological,  
739 developmental and growth processes in plants may rapidly occur in the near future.

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745

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**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, ? indicates not known, (?) indicates possibility based upon indirect evidence. Note ALMTs do not necessarily respond to all these factors, similarly for R-type channels.

**Fig 2 (a and b) - Schematic representation of GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) structure.** **a)** Transmembrane topology reveals that each subunit is composed of 4 hydrophobic transmembrane domains (TM1-4), a large extracellular ligand binding NH<sub>2</sub> region with a disulphide bond characteristic of the family of cys-loop receptors and a short barely extruding COOH terminus. 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 3 interact with neighbouring subunits, TM2 faces the lumen of the ion channel while TM4 is anchored in the lipid membrane. **(c and d) Schematic representation of predicted topology models of wheat (Ta)ALMT1.** **c)** Model proposed by Motoda et al., 2007 predicts both N and C termini to be extracellular with 6 transmembrane domains. The residues important for GABA binding reside at the end of 6 TM and are indicated by the red arrow. **d)** The second model proposed by Dreyer et al., 2012 predicts that in addition to 6 transmembrane domains, the large N terminus may contain another transmembrane domain (shown in grey). Further 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 are indicated by the red arrow and the highly conserved WEP motif and phosphorylation site (S384) are also shown.

**Fig 3 Sequence alignment of rat GABA<sub>A</sub>  $\alpha$  subunit with wheat TaALMT1.** Residues important for GABA sensitivity indicated by an \* in the rat GABA<sub>A</sub>  $\alpha$  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 coverage 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.

**Fig 4 One 3D model of TaALMT1 protein computed from evolutionary sequence variation using the EvFold web portal (<http://evfold.org/evfold-web/evfold.d>).** **a)** Top 50 modeled contacts computed from co-evolution of residue pairs from 3,688 alignments using TaALMT1 as input with overall E value of 10<sup>-5</sup>. 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 hot-spot 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 6 transmembrane (TM) domains (orange, cyan and red) with N-terminus first TM denoted orange and the 6<sup>th</sup> 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 (asterix) and two residues at the N-terminus and start of 1<sup>st</sup> TM that showed significant evolutionary coupling (R40 and Y96 among a total of 7 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.

**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. mollendorffii*, *T. aestivum*, *C. reinhardtii*, *V. carteri* and GABA<sub>A</sub>  $\alpha$  subunit from *Rattus norvegicus* (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., 2012. The sequence alignment was used to generate consensus sequence and sequence log using Geneious 9.0.4. The scale bar to the left of the graph shows minimum and maximum coverage 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.

Fig. 1

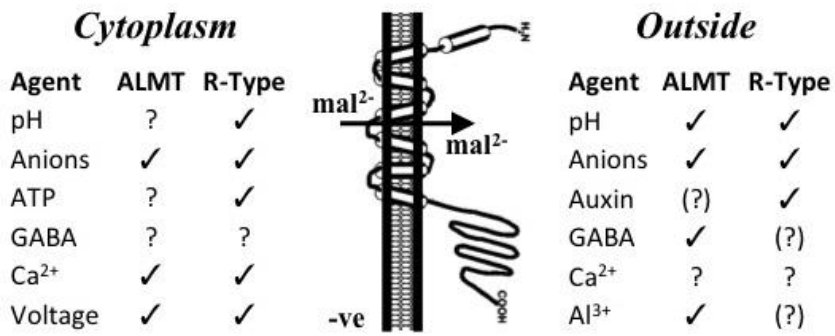


Fig. 2

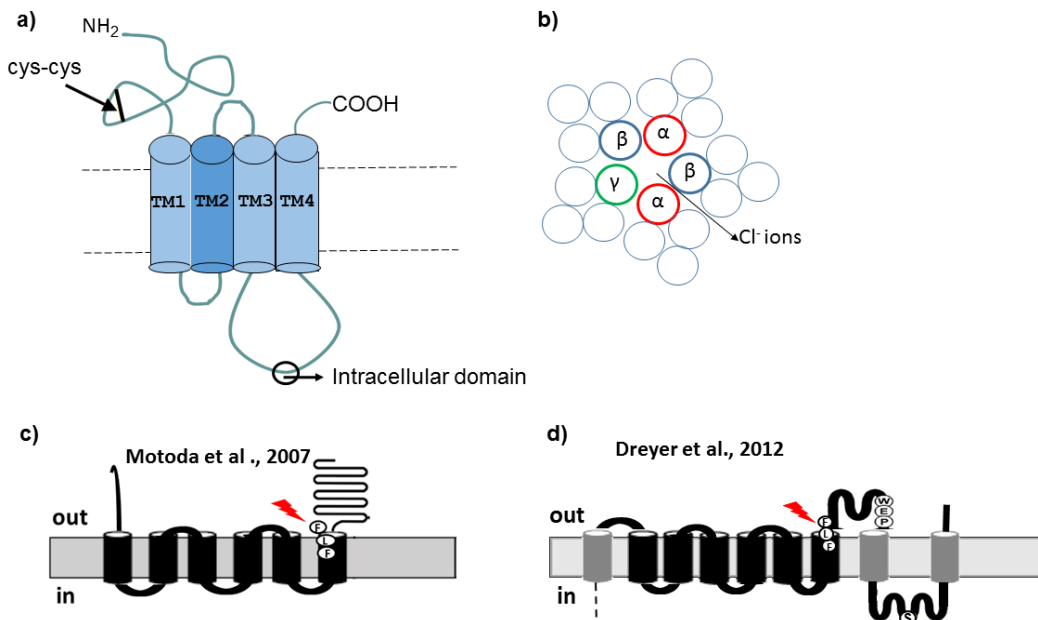
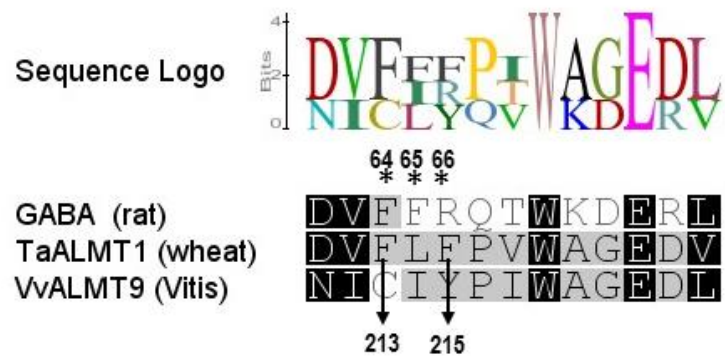


Fig. 3



**Fig. 4**

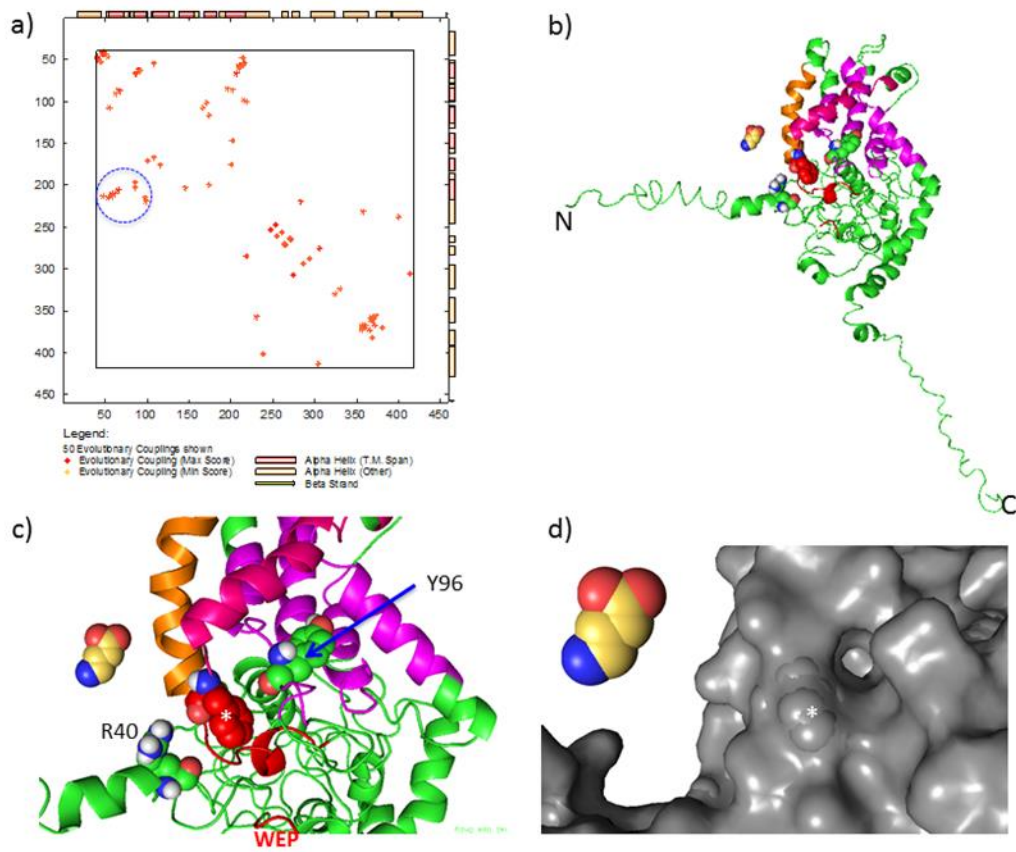
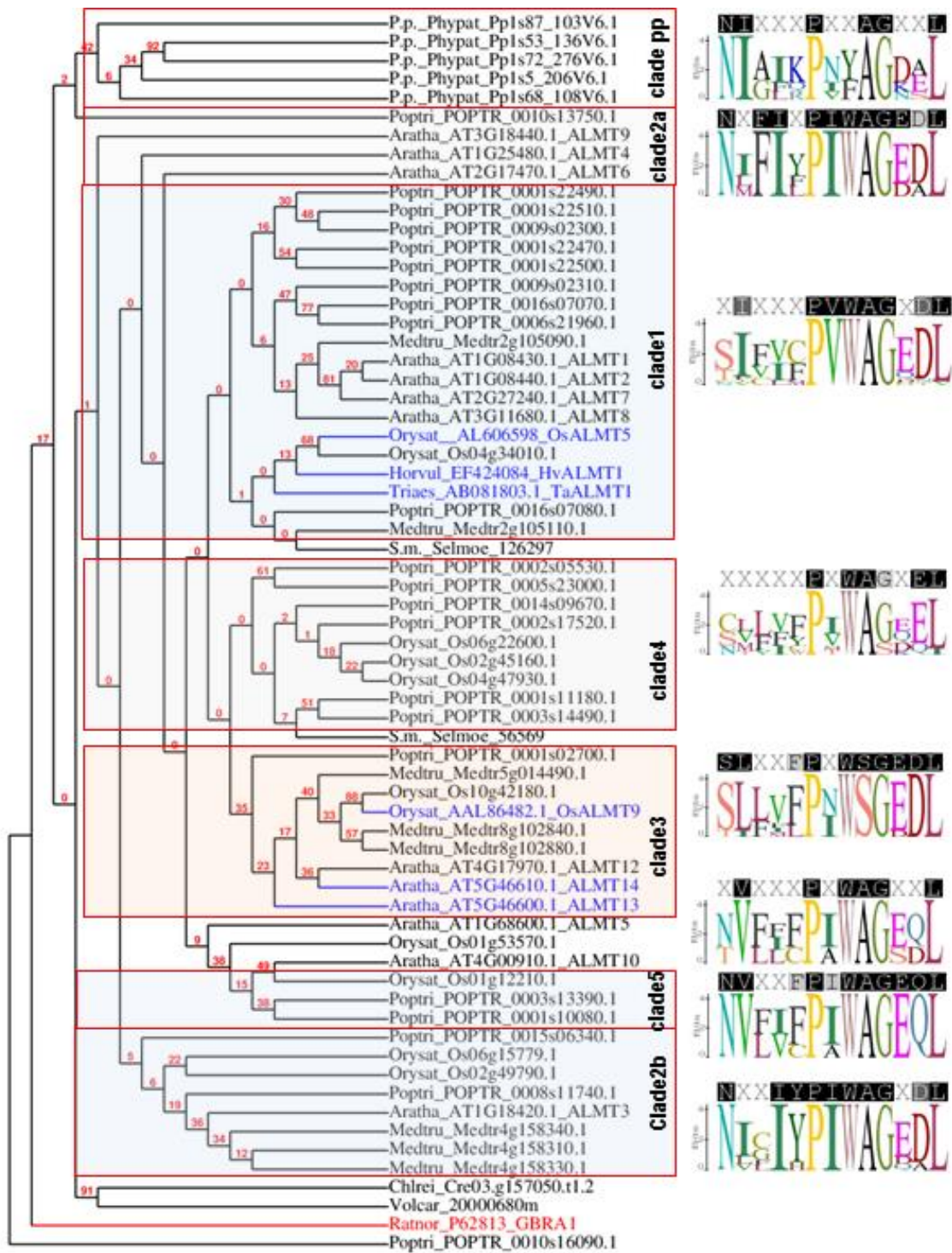


Fig. 5



**Table 1 GABA distribution in different plant organs and species.** GABA has been found in all organs in plants, including embryo, cotyledon, roots, shoot, flowers, fruit, nodule, xylem and phloem. FW = fresh weight, DW = dry weight, \*GABA gradient exists from top to bottom in pistils of flowers.

Species	Organs	GABA concentration	References
<i>Arabidopsis thaliana</i>	root	~ 0.40-0.1 $\mu\text{moles.g}^{-1}$ FW / ~8 $\mu\text{moles.g}^{-1}$ DW	[19,160,161,170,179,188,273-275]
	shoot	~ 0.03-1 $\mu\text{moles.g}^{-1}$ FW / <1 $\mu\text{moles.g}^{-1}$ DW	
	flowers	~ 0.2 $\mu\text{moles.g}^{-1}$ FW	
<i>Nicotiana tabacum</i>	pistil*	~0.6-4 $\mu\text{moles.g}^{-1}$ FW	[261]
	shoot	~0.2-1 $\mu\text{moles.g}^{-1}$ FW	[209,273]
	root	< 0.2 $\mu\text{moles.g}^{-1}$ FW	[276]
	seedling	~25 $\mu\text{moles.g}^{-1}$ FW	
<i>Nicotiana sylvestris</i>	leaf	~10 $\mu\text{moles.g}^{-1}$ FW	[277]
<i>Brassica napus</i>	root	~0.5 $\mu\text{moles.g}^{-1}$ FW / ~3.6 $\mu\text{moles.g}^{-1}$ DW	[163]
	leaf	~1.30 $\mu\text{moles.g}^{-1}$ FW / ~1.1 $\mu\text{moles.g}^{-1}$ DW	[278]
<i>Oryza sativa</i>	calli	~0.2-0.3 $\text{nmoles.g}^{-1}$ FW	[260,279,280]
	root	~0.5-1 $\mu\text{moles.g}^{-1}$ FW	
	shoot	<0.5-1 $\mu\text{moles.g}^{-1}$ DW	[187,281]
	kernel	~0.01-0.12 $\mu\text{moles.g}^{-1}$ FW	[282]
	embryo	<5 $\mu\text{moles.g}^{-1}$ FW	[283]
<i>Glycine max</i>	xylem	~ 100-160 $\mu\text{M}$	[199,284,285]
	leaf	~0.05-0.4 $\mu\text{moles.g}^{-1}$ FW	
	root	~0.1 $\mu\text{moles.g}^{-1}$ FW	
	nodule	~1.5 $\mu\text{moles.g}^{-1}$ FW	[180]
	seedling	<1 $\mu\text{moles.g}^{-1}$ FW	
	cotyledon	~25 $\mu\text{moles.g}^{-1}$ DW	
	embryo	~15 $\mu\text{moles.g}^{-1}$ DW	
<i>Medicago sativa.</i>	root	~0.4 $\mu\text{moles.g}^{-1}$ FW	[196]
	nodule	~2.4 $\mu\text{moles.g}^{-1}$ FW	[251]
	phloem	~1.4 $\text{nmoles.g}^{-1}$ FW	
<i>Solanum lycopersicum</i>	fruit	~0.5-40 $\mu\text{moles.g}^{-1}$ FW	[286-288]
	leaf	~3-5 $\mu\text{moles.g}^{-1}$ FW	[8,30,269]
<i>Triticum aestivum</i>	root	~2-4 $\mu\text{moles.g}^{-1}$ FW	[18,289]
	seedling	~0.02 $\mu\text{moles.g}^{-1}$ FW	[178]
<i>Hordeum vulgare</i>	seedling	~0.02 $\mu\text{moles.g}^{-1}$ FW	[178]
<i>Eriobotrya japonica</i>	fruit	~0.15-0.35 $\mu\text{moles.g}^{-1}$ FW	[237]
<i>Cucumis melo</i>	root	~0.25 $\mu\text{moles.g}^{-1}$ FW	[290]
<i>Vicia faba</i>	bean	<10 $\mu\text{moles.g}^{-1}$ DW	[291]
<i>Vitis vinifera</i>	berry	~1.4 $\mu\text{moles.g}^{-1}$ FW	[292]
<i>Comellia sinesis</i>	leaf	~15 $\mu\text{moles.g}^{-1}$ DW	[293]
<i>Phaseolus vulgaris</i>	leaf	~4.4-9 $\mu\text{moles.g}^{-1}$ DW	[198]
<i>Pisum sativum</i>	nodule	<1.5 $\mu\text{moles.g}^{-1}$ FW	[294]
<i>Caragana intermedia</i>	root	<0.05 $\mu\text{moles.g}^{-1}$ FW	[295]

**Table 2 Effect of mutations on residues important for GABA binding.**

Name	Wild Type Residue	Affinity (EC <sub>50</sub> μM)	Mutation	EC50 (μM)
GABA <sub>A</sub> α1 (rat)	F <sup>64</sup>	594	F <sup>64</sup> to C <sup>64</sup>	72.8
	F <sup>65</sup>	19	F <sup>65</sup> to C <sup>65</sup>	2.34
	R <sup>66</sup>	2610	R <sup>66</sup> to C <sup>66</sup>	320
TaALMT1	F <sup>213</sup>	3.4	F <sup>213</sup> to C <sup>213</sup>	1000
	F <sup>215</sup>	3.4	F <sup>213</sup> /F <sup>215</sup> to C <sup>213</sup> /C <sup>215</sup>	1853

**Table 3a Regions in other proteins that may have a role in GABA binding.** Amino acid regions identified using BLAST search using consensus sequence “DVFXXXXWXXEXL”. (Coverage above 80% only listed below).

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

**Table 3b Regions in Arabidopsis proteins that may have a potential role in GABA binding.** Amino acids identified using BLAST search with GABA binding motif “DVFFXPTWXGEXL”. (Coverage above 90% only listed).

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



**Table 4 Overview of drugs tested as agonists, antagonists or modulators of GABA receptors in animals and plants**

<b>Drug</b>	<b>Source</b>	<b>Action on animal GABA receptors</b>	<b>Effect on animal GABA receptors</b>	<b>References</b>	<b>Tested in plants</b>	<b>Effects in plants</b>	<b>References</b>
Bicuculline	<i>Dicentra cucullaria;</i> <i>Corydalis sp.</i> , <i>Adlumia sp.</i>	Competitive antagonist	Mimics epilepsy	[296]	Yes	Ameliorates the inhibition of anion flux by GABA	[18]
Picrotoxin	<i>Anamirta cocculus</i>	Non-competitive antagonist	Blocker for the GABA <sub>A</sub> receptor	[296]	No	Unknown	
Bilobalide and Ginkgolides	<i>Gingko biloba</i>	Negative allosteric modulator	Acts on GABA <sub>A</sub> receptors and GABA <sub>A</sub> -rho receptors	[296,297]	No	Unknown	
Muscimol	<i>Amanita muscaria</i>	Agonist	Sedative-hypnotic and dissociative psychoactivity	[298,299]	Yes	Inhibits anion flux	[18]
GABA	Plants - Chocolate, tea wine	Agonist	Reducing neuronal excitability	[110,300]	Yes	Inhibits anion flux	[18]
Flavanoids	Red wine, Vegetables, Green tea	Modulators-Benzodiazepine binding	Anti allergic/anti inflammatory, anti microbial/anti oxidant	[297]	No	Unknown	
$\alpha$ pyrones	<i>P. methysticum</i> , cinnamon, cloves, and ginger,	Positive modulators	Facilitates cell to cell communication	[297]	No	Unknown	
Apigenin	<i>Matricaria recutita</i> (Chamomile), parsley, celery, celeriac	Anxiolytic properties	Possible chemo-preventive role in Leukemia	[301-303]	No	Unknown	
Flumazenil	Synthetic	Benzodiazepine receptor antagonist	Anaesthesia reversal Benzodiazepine overdose	[304,305]	No	Unknown	

Amentoflavone	St. John's wart <i>Ginkgo biloba</i>	influences G-protein-coupled receptors, for serotonin, dopamine etc	Anti cancer/Anti malarial	[306]	No	Unknown
Baclofen	Synthetic	Mainly GABA <sub>A</sub> receptor agonist	Spasticity/Addiction	[42,307,308]	Yes	Increased GABA mediated promotion of growth in <i>Lemna minor</i> [22]
Gabaculine	<i>Streptomyces toyacaensis</i>	Irreversible GABA- $\alpha$ Ketoglutaric acid Transaminase inhibitor, GABA reuptake inhibitor	Research only purposes-increases GABA levels	[309,310]	No	Unknown
Vigabatrin	Synthetic	GABA-T inhibitor	Treatment of epilepsy	[111,311,312]	Yes	Increases endogenous GABA concentrations [18]
GHB ( $\gamma$ -Hydroxybutyric acid)	Endogenous -plants & animals	Naturally occurring neurotransmitter	General anaesthetic, insomnia, narcolepsy, alcoholism, recreational drug etc	[313-316]	No	Unknown
Barbiturates	Synthetic	Central nervous system depressants	Anxiolytic, sedative, hypnotic	[317-319]	No	Unknown
Benzodiazepines	Synthetic	Inhibit GABA <sub>A</sub> receptors	Anxiolytic, sedative, hypnotic, muscle relaxant	[320,321]	No	Unknown