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1   **Linking metabolism to membrane signalling: the GABA-malate connection.**

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Abstract

13   GABA concentration increases rapidly in tissues when plants encounter abiotic

14   or biotic stress, and GABA manipulation affects growth. This coupled to GABA's

15   well-described role as a neurotransmitter in mammals led to over a decade of

16   speculation that GABA is a signal in plants. The discovery of GABA-regulated

17   anion channels in plants provides compelling mechanistic proof that GABA is a

18   legitimate plant-signalling molecule. Here, we examine research avenues

19   unlocked by this finding, and propose that these plant 'GABA receptors' possess

20   novel properties ideally suited to translating changes in metabolic status into

21   physiological responses. Specifically, we suggest they have a role in signalling

22   altered cycling of Tricarboxylic Acid (TCA) intermediates during stress via

23   eliciting changes in electrical potential differences across membranes.

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Glossary box

ABA – abscisic acid

ACC synthase – 1-aminocyclopropane-1-carboxylate synthase, a rate-limiting step in the biosynthesis of ethylene

Al^{3+} - trivalent aluminium cation

ALMT - Aluminium activated malate transporter, forms functional protein from one protein isoform

AKGDH – α-ketoglutarate dehydrogenase

ATP – Adenosine triphosphate

Baclofen – a specific $GABA_B$ receptor agonist

Bicuculline – a plant-derived alkaloid that is a 'specific' mammalian $GABA_A$ receptor competitive antagonist, which associates with the GABA binding site but does not activate the channel, instead it prevents GABA from activating the channel

C – Carbon

Carboxylate (ion) – anionic conjugate base of a carboxylic acid of general formula $RCOO^-$

GABA – γ-aminobutyric acid, a non-proteinogenic amino acid and zwitterion; acts as an inhibitory neurotransmitter in animals

$GABA_A$ receptor –mammalian ionotropic GABA receptor formed from the multimerisation of various combinations of α, β and γ subunits; chloride channel that is opened by the agonist GABA. This hyperpolarises the membrane potential to reduce membrane excitability

$GABA_{A-\rho}$ – rho (ρ) sub-class of $GABA_A$ receptors also known as $GABA_C$ receptors. Similar to ALMT they can form a functional channel using only one subunit form

GABA shunt – the main pathway of GABA metabolism, which bypasses reactive oxygen species (ROS) sensitive steps of the TCA cycle (OGDH and AKGDH). The GABA shunt consists of the enzymes GAD, GABA-T and SSADH

GAD – glutamate decarboxylase, the cytosolic enzyme that synthesizes GABA via decarboxylation of glutamate, this process absorbs a proton and releases CO_2; GAD is stimulated by Ca^{2+}/Calmodulin and has an acidic pH optimum

GABA-T – GABA transaminase, mitochondrial enzyme encoded by the *pop2* allele in *Arabidopsis thaliana*, converts GABA into SSA

GABP – mitochondrial GABA carrier

GAT1 – plasma membrane localised high affinity GABA transporter

GHB – α-hydroxybutrate, alternative breakdown product of SSA, particularly under stress via action of cytosolic and plastidial glyoxylate reductase

GDH – glutamate dehydrogenase

66 GLR – glutamate receptor-like proteins, the homolog of ionotropic glutamate
67 receptors in animals (iGluR), which mediate excitatory neuronal signals

68 GTP – guanosine triphosphate

69 Malate – $C_4H_6O_5$; dissociates into malic acid consisting of the carboxylate malate
70 anion, and H^+; the form of malate is pH sensitive with a pKas of 3.4 and 5.2

71 Muscimol – analog of GABA, and 'specific' and 'diagnostic' $GABA_A$ receptor
72 agonist, derived from the mychorrhizal fungus *Amanita muscaria*

73 PM – plasma membrane

74 Picrotoxin – non-competitive $GABA_A$ receptor antagonist that inhibits channel
75 activity through occluding the pore and open channel block. It is a plant alkaloid
76 derived primarily from *Anamirta cocculus*

77 N – Nitrogen

78 OGDH – 2- oxoglutarate dehydrogenase

79 ProT – low affinity plasma membrane localised proline transporter that is also
80 permeable to GABA

81 TCA cycle – tricarboxylate acid, also known as the citric acid or Krebs cycle

82 ROS – reactive oxygen species

83 SSA – Succinic semialdehyde, converted into succinate by SSAH

84 SSADH – Succinic semialdehyde dehydrogenase – mitochondrial enzyme that
85 converts SSA into succinate for entry into the TCA cycle

86

Outstanding questions

- How does GABA directly regulate ALMT activity – through direct binding and channel gating, or through GABA occlusion of the pore (i.e. open channel block)? *A rapid GABA-induced reduction in anion efflux through ALMT has been observed but the mechanism by which this occurs has not been described.*

- Where is the putative GABA binding region within the ALMT protein structure? Is it on the inside or outside of the cell or accessible to both sides? *The topology of the ALMT is under debate; the topology has implications for where the putative GABA binding region is located.*

- Do functional ALMT consist of multiple monomeric or multimeric subunits, if so can these be heteromeric as well as homomeric? *Many mammalian GABA receptors are composed of multiple subunits with the GABA binding site formed between adjacent subunits. Single ALMT genes form a functional protein when expressed individually in X. laevis oocytes but it is unclear whether the channel is composed of multiple subunits and/or whether the putative GABA binding site can occur within a monomer.*

- Are there additional regions of the ALMT associated with GABA efficacy and are there additional GABA sensitive proteins?

- How does GABA exit the cell? *The protein responsible for efflux of GABA has not been confirmed.*

- How do the other signals regulate ALMT/R-type anion channel activity and how do these signals interact with GABA (i.e. Aluminium, pathogens, ABA, ethylene)? *As multiple signals regulate ALMT activity they can be viewed as an important regulator of membrane potential – how multiple signals converge and interact on the one protein is unclear.*

- Are there transcriptional signals downstream of GABA regulation of ALMT activity?

- Does GABA directly regulate the activity of other transporters or is the regulation of other ion fluxes due to indirect regulation via ALMT activity?

- Is it possible to develop a biosensor for GABA for recording in vivo GABA concentrations in real time?

122   TRENDS BOX

123

124   • GABA, the non-protein amino acid regulates the activity of ALMTs
125   (Aluminium activated anion transporters), these, like mammalian $GABA_A$
126   receptors are anion channels that alter the electrical potential across
127   membranes.
128   • ALMT are a multigenic protein family found in all plants that are involved
129   in multiple physiological processes; contrary to their name most ALMT
130   are not activated by aluminium, instead they are activated by anions and
131   negatively regulated by GABA.
132   • ALMTs share little homology to mammalian $GABA_A$ receptors; the only
133   region of similarity so far identified is a putative GABA binding domain 12
134   amino residues in length.
135   • Site directed mutagenesis of a phenylalanine in this motif renders wheat
136   ALMT1 unresponsive to GABA, but otherwise unchanged in its properties.
137   • Regulation of ALMT by GABA and malate provides a link between the TCA
138   cycle and membrane signalling.

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Main text

(re-)Defining the roles of GABA in plants

GABA is an important **C:N** metabolite produced by plants and animals; it is a significant source of succinate for the **TCA cycle** (see glossary). In plants, it has also been proposed to have roles in C:N balance [1, 2], the regulation of cytosolic pH [3, 4], protection against oxidative stress [5, 6], salt tolerance [7], plant microbe interactions [8-10], defence against insect attack [11], and as an endogenous signalling molecule [12-14]. Speculation that GABA acts as a plant signal was originally spawned over a decade ago, and has been reiterated frequently, following the convergence of multiple strands of evidence [6, 15, 16]: i) GABA concentration increases rapidly in plant tissues upon any of a multitude of abiotic (anoxia, heat, cold, salt, drought, mechanical) or biotic stresses (herbivory, pathogens, viruses)[12, 17, 18] ; ii) animals contain both ionotropic and metabotropic GABA receptors [19, 20], and GABA is a key neurotransmitter [21, 22] and intercellular signal in immune cells [23]: iii) plants contain **GLR**s that are involved in regulation of growth, wound signalling and plant defence signalling [24-26]: iv) some GABA receptor agonists (**baclofen**) and antagonists (**bicuculline** and **picrotoxin**) produced a phenotypic response in plants [15]; v) pollen tube growth to the ovary is directed by a GABA gradient [13, 27]; and, vi) the presence of GABA binding sites on plant membranes [28]. For GABA to constitute a signal in plants a receptor would be needed, but no homologs of animal GABA receptors are present in Arabidopsis [6]. It was hypothesised that GLRs could fulfil a GABA receptor role through their action as ligand gated ion channels [6], some of which are Ca^{2+} permeable [24, 29] and are activated by a range of amino acids [30]; however, GABA does not appear to have been tested

169 [30, 31]. Instead, it was recently found that GABA regulated **ALMT** channel

170 activity, and that this resulted in changes in plant growth [32]. This implicates

171 ALMT proteins as prime 'plant GABA receptor' candidates.

172

173 In this opinion article we briefly highlight the findings of Ramesh et al. (2015)

174 and discuss their ramifications. In particular, we focus on how the activation of

175 ALMT by several TCA cycle intermediates and their inhibition by GABA, positions

176 ALMT as likely metabolic sensors that are able to decode environmental

177 responses into physiological outputs. Furthermore, we propose a series of

178 unanswered questions and research streams that stem from the discovery of

179 GABA-regulated anion channels in plants.

180

181 **The discovery of GABA-regulated anion channels in plants**

182 Under acidic conditions, when **Al^{3+}** is available in the soil solution, free Al^{3+}

183 causes significant damage to root apical cells, reducing root elongation, nutrient

184 and water uptake, and crop yield [33, 34]. When cells of Al^{3+}-tolerant plant roots

185 come into contact with Al^{3+}, **carboxylate** exudation is stimulated. This

186 carboxylate chelates the Al^{3+} in the apoplast and rhizosphere, which prevents

187 damage to roots [33]. Whilst studying this process in wheat (*Triticum aestivum,*

188 *Ta*), Ramesh et al. (2015) found that under conditions where root carboxylate

189 (**malate**) efflux was high i.e. in acidic conditions with Al^{3+} present or under

190 alkaline conditions, tissue GABA concentration was low and this coincided with

191 sustained root growth [32]. In contrast, when malate efflux was low, GABA

192 concentration was high (i.e. under acidic conditions when Al^{3+} was absent) [32].

193 Treatment of roots with GABA inhibited malate efflux and abolished Al^{3+}

194   tolerance [32]. Intriguingly **muscimol** inhibited root malate exudation and Al^{3+}

195   tolerance, whilst bicuculline attenuated muscimol and GABA inhibition of root

196   malate efflux [32]. These results indicated similarities between the mechanism of

197   GABA regulation of anion flux across plant and animal cell membranes [35].

198

199   When the pharmacological profile of TaALMT1, the source of wheat root malate

200   exudation stimulated by Al^{3+} – and the major source of wheat Al^{3+} tolerance –

201   was examined in heterologous expression systems it mirrored the results found

202   in wheat roots [32]. The affinity for TaALMT1 GABA-regulation was found to be

203   in the low micromolar range [32], similar to that for animal $GABA_{A-\rho}$ receptors

204   and some $GABA_A$ receptors [19]. Sequence comparison between TaALMT1 and

205   ion channels used to construct a $GABA_A$ receptor model [36] revealed a shared

206   motif of 12 amino residues [32]. This motif contained residues that have been

207   associated with GABA binding in $GABA_A$ receptors [37]. Mutagenesis of this motif

208   in TaALMT1 reduced the EC_{50} for GABA from ~ 1 μM to >1 mM, but preserved

209   other properties of the channel such as Al^{3+} activation [32]. A fluorescent

210   muscimol-conjugate associated with membranes of *Xenopus laevis* expressing

211   wildtype TaALMT1 but not when the mutagenized GABA unresponsive TaAMLT1

212   was expressed; wheat roots that highly expressed *TaALMT1* also fluoresced

213   highly when exposed to the conjugate whereas the signal was much reduced in

214   roots of Al^{3+} sensitive wheat [32]. This suggests that muscimol and GABA bind to

215   this motif in ALMT.

216

217   In mammals, GABA activates Cl^- channels in the mature central nervous system

218   and immune cells leading to Cl^- influx, membrane hyperpolarisation and a

219   reduction in membrane excitability [38]. GABA regulation of ALMT results in a

220   relative hyperpolarisation of the membrane potential and an inhibition of

221   growth [32]. The similarity in how plants and animals respond to GABA suggests

222   that convergent evolution has occurred, unless this motif was recruited

223   independently into both sets of proteins from a common source [35].

224

225   The significance of ALMT sensitivity to GABA becomes evident with the

226   knowledge that ALMT are present in all plants and form a multigenic family;

227   Arabidopsis has fourteen members, rice has nine and grapevine twelve [39].

228   *ALMT* are expressed throughout plant tissue with some having discrete

229   expression patterns, and the majority of ALMT contain the putative GABA

230   binding motif [32]. Despite their name Al^{3+} does not activate most ALMT, instead

231   all ALMT members examined so far are activated by anions, in particular malate,

232   and they are involved in a range of physiological processes including stomatal

233   movement, pollen tube growth, nutrition, and grape berry and tomato ripening

234   [39]. Arabidopsis ALMT12 carries the majority of rapid-type (R-type)/(Quickly

235   activating-QUAC) anion current in guard cells, so it has been speculated that

236   other ALMT may also carry other R-type anion currents found in most cell types

237   [40] [39]. The moniker ALMT was adopted following the properties of the first

238   member identified, TaALMT1, so given the above the proposal that the ALMT

239   family should be renamed is a valid suggestion [32, 39].

240

241   **The when, why and how of GABA-gated ALMT?**

242   Plants alter their internal GABA concentration in response to environmental

243   changes within seconds; GABA can also follow daily rhythms or exhibit sustained

244 increases over days [6, 16, 41, 42]. A frequent immediate response to

245 environmental stress is an increase in cytosolic Ca^{2+} concentration, which

246 activates **GAD,** resulting in GABA synthesis; this increase in GABA concentration

247 is then capable of reducing ALMT activity (Figure 1). This bodes well for GABA

248 being a signal; however, many questions remain.

249

250 *Linking GABA-regulated membrane potential to transcription and phenotype?* A

251 consistent phenotype downstream of the disruption of GABA metabolism, or the

252 application of exogenous GABA is altered growth. This has been observed for

253 pollen tubes, roots and hypocotyls [27, 43]. The regulation of membrane

254 potential is known to constitute a signal in many cell types. Stress affects both

255 stomatal aperture and pollen tube growth; in both stomatal guard cells and

256 pollen tubes the control of anion fluxes – and membrane potential – is essential

257 for the control stomatal movement and growth respectively [40, 44]. *ALMT* are

258 expressed in both guard cells and pollen tubes, and have a confirmed role in

259 stomatal aperture control [45] [40, 46, 47]. Whether GABA regulation of ALMT

260 activity occurs in these systems and is a signal is an active area of research.

261

262 GABA has been proposed to affect the flux of ions other than anions. Influx of

263 Ca^{2+} via channels localised to pollen tubes was proposed to increase following

264 GABA treatment in tobacco as a component of pollen tube guidance to the ovary

265 [16, 43]. Whereas GABA pre-treatment followed by H_2O_2 application to barley

266 roots (in lieu of hypoxia) resulted in less cell death, a stimulation of net Ca^{2+}

267 efflux and reduced net K^+ efflux [48]. As sustained K^+ release across a membrane

268 cannot occur without the movement of a balancing charge, GABA may be

269   affecting K^+ flux indirectly by inhibiting anion release via an ALMT. GABA-

270   regulation of ALMT is also likely to affect Ca^{2+} channel activity via provoking

271   changes in membrane potential [32] (Figure 1). How specific GABA-elicited

272   changes in transporter activity and membrane potential affect gene transcription

273   is unclear. However, expression of 14-3-3 proteins, which have a range of targets

274   including pumps, channels and transcription factors, is reduced in Arabidopsis

275   seedlings by GABA in an ethylene and ABA dependent manner [49]. GABA also

276   increases **ACC synthase** expression and ethylene production in sunflower [50]

277   and considering ethylene can also reduce TaALMT1-dependent malate efflux

278   [51] there appears to be cross-talk between GABA and ethylene signalling. When

279   GABA was applied in the absence of Ca^{2+} and under C or N limitation cell wall

280   synthesis genes were inhibited, in addition to a change in expression of a small

281   subset of genes that also respond to abiotic stress, inorganic substances and

282   others that are involved in C or N metabolism [52, 53]. In another study both

283   malate and citrate induced changes in transcription, which included ***GABA-T*** and

284   *ALMT* genes [54]. As so few transcripts were altered in Arabidopsis grown in

285   liquid culture following 1 mM GABA treatment (eighty-nine) it was argued that a

286   broad signalling role for GABA is unlikely [53]. However, it is possible that

287   signals may have been masked by analysing transcription in multiple cell types

288   or under particular experimental conditions.

289

290   *Topology and the regulation of local GABA concentration*. The current model for

291   TaALMT1 topology, and its rapid inhibition by external GABA, suggests that the

292   putative GABA binding motif is localised on the extracellular face of the

293   membrane but a role for cytosolic GABA in regulating plasma membrane or

11

294   tonoplast localised ALMT cannot be excluded [32]. GABA regulation of ALMT was

295   not completely abolished by mutagenesis of the proposed GABA binding motif so

296   there is scope for other regions of the protein to interact with the motif or for

297   multiple GABA binding sites. Of all the amino acids, GABA shows the highest

298   efflux from wheat roots but interestingly shows a similar amount of influx [55].

299   Such an apparent futile cycle may be justified energetically if GABA was

300   important for cell-to-cell communication or biotropic interactions. The identified

301   uptake transporters for GABA (**GAT1** and **ProT**) may be involved in efflux but

302   this is unlikely for GAT1 given its probable mechanism of proton co-transport

303   [56], and the fact that GABA efflux is passive [57]. Recently, the role of GAT1 in

304   GABA influx into cells was further reinforced by use of Arabidopsis *gat1*

305   knockout mutants [58]. Feeding GABA to C deficient plants resulted in GABA

306   influx into the cells and the TCA cycle of wildtype plants whereas this did not

307   occur in *gat1* mutants [58].

308

309   GABA can be found in micromolar concentrations in root exudates and the

310   apoplast [55], but for it to act as a signal it will need to have local concentrations

311   in the vicinity of ALMT strictly controlled. An initial argument against GABA

312   being a signal in animals was the presence of millimolar concentrations of GABA

313   in the bulk tissue [59, 60]. The same argument could be applied against GABA

314   being a plant signal; however, evidence already exists for tight control of plant

315   tissue GABA concentrations including the existence of micromolar GABA

316   gradients in floral tissue [6, 13, 16]. Further investigation of pharmacology will

317   greatly assist the characterisation of GABA signalling in plants as it did for

318   mammalian GABA receptors [32, 59]. Another useful innovation will be the

319 ability to detect GABA concentration with both cellular and apoplastic

320 resolution; this has been performed in plants to date using a commercial GABA

321 antibody in fixed tissue [13]. A GABA sensor that allows time-resolved

322 micromolar spatial resolution in living tissue would be a great benefit to explore

323 how GABA compartmentation is regulated. Some progress has occurred using

324 whole-cell patch clamp with human embryonic kidney cells expressing GABA

325 receptors [61] or GABA imprinted electrodes [62].

326

327 *Do other metabolites regulate ALMT?* It has been proposed that the role of

328 TaALMT1 in sustained anion efflux may be a specialisation in the family [39].

329 Instead, the ALMT family generally appear to be activated by malate and

330 inhibited by GABA [32], but these metabolites appear to be one of a number of

331 signals that act upon ALMT to fine tune cellular responses to environmental or

332 metabolic conditions. For instance, ALMT/R-type anion channels are also

333 negatively regulated by niflumate, nucleotides including **ATP** and **GTP**, voltage,

334 ethylene (indirectly via GABA?), and positively regulated by ABA (indirectly?),

335 phosphorylation, malate, succinate, fumarate, pathogens, voltage and a range of

336 inorganic ions [32, 39]. Considering the wide range of compounds already

337 known to affect ALMT activity other regulators are likely to exist. Candidate

338 compounds included **GHB**, other amino acids, other carboxylates and

339 compounds related to GABA metabolism [32, 35, 63]. It is tempting to speculate,

340 despite the lack of a nervous system, plants could propagate long distance

341 electrical signals through ALMT in a similar fashion to that observed recently for

342 GLRs, through the interaction with the gating signals of GABA- and carboxylate

343 (malate); such a signal could mediate long-distance as well as local changes in

344   transcription. In Figure 1 we propose a model of how the properties of ALMT

345   make it suitable as a signalling interface between carbon metabolism and

346   physiological responses by the plant.

347

348   The flux of C intermediates, NADH and ATP production during aerobic

349   respiration does not always occur in a cyclical fashion through the classical

350   components of the TCA cycle [64]. When excessive **ROS** are induced by

351   environmental conditions **OGDH** and **AKGDH** are inhibited and 2-oxoglutarate is

352   transported out of the mitochondria into the cytoplasm where it is converted to

353   GABA via the combined action **GDH** and GAD. GABA is then shunted back into the

354   mitochondria where it is converted into SSA and finally succinate (Figure 1). The

355   fact that there are multiple membrane transport steps needed suggests that

356   there is a reason for compartmentation of GABA metabolism – a plausible

357   reasons for this could be the accessibility of cytoplasmic GABA to the plasma

358   membrane so it can be used as a signal, in addition to its role as a metabolite in

359   the mitochondria.

360

361   **Concluding remarks.**

362   The identification of GABA-gated anion channels in plants provides a convincing

363   'plant GABA receptor' candidate and appears to provide a definitive answer in

364   the debate of whether GABA can be a plant-based signal or whether it is just a

365   metabolite. However, this finding also poses many more questions, many of

366   which are covered here (Outstanding questions box). To rouse research activity

367   and set a research agenda, we have also proposed a model of how GABA and

368   carboxylate concentration, transport and action might co-ordinate to signal plant

369   metabolic status (Figure 1). Testing this model should help to further explain

370   how and when GABA signalling occurs in plants.

371

Acknowledgments

376

377   **Figure 1. Model proposing how ALMT could sense and signal metabolic**

378   **status.** The regulation of ALMT activity on the PM by carboxylate anions, in

379   particular malate (positive, blue arrow), and GABA (negative, red arrow)

380   provides a mechanism by which changes in cell metabolism could lead to voltage

381   changes (ΔVm); this could convey cell metabolic status to adjacent cells. GABA

382   inhibits the ALMT channel with high affinity (1 µM), whereas malate activation

383   has a lower affinity (1 mM), these properties may lead to transient activation of

384   ALMTs. GABA acts on the outside of the channel in heterologous systems, but

385   GABA-binding sites may also exist on the cytoplasmic side, or be accessible from

386   both sides [32]. How GABA effluxes from the cell is not known, but there is a

387   high affinity uptake transporter (AtGAT) [16, 63], which may be critical in

388   regulating apoplasmic GABA concentrations. GABA and malate are connected in

389   metabolism via pathways through the tricarboxylic acid (TCA) cycle and the

390   GABA shunt [6, 16, 54, 63, 64]. A likely negative correlation between TaALMT1

391   activation and the concentration of GABA [32] may reflect the metabolic flux

392   through the GABA shunt to malate, or be associated with the regulation of GAD,

393   which produces GABA from glutamate [4]. Negative regulation of malate flux

394   from cells will result in the conservation of an energy rich C source for the cell to

395   use during stress.

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Metabolism-To-Membrane

Pyruvate → Pyruvate

Cytoplasm

OAA → OAA

Acetyl CoA

Citrate → Citrate

Malate ← Malate

TCA cycle

Isocitrate

Fumarate

2-oxoglutarate

Succinate

Succinic semialdehyde

Glutamate

GABA-T

GAD

GABA

Mitochondria

To other transport, Ca^{2+}, H^+

ΔVm

ALMT

?

GAT

pm

Apoplasm

Malate

GABA

Extracellular signals to and from other cells via ALMTs