

Regulation of Arabidopsis 14-3-3 gene expression by GABA

Running title: GABA regulation of gene expression.

MURIEL LANCIEN AND MICHAEL R. ROBERTS*

*Department of Biological Sciences, Lancaster Environment Centre, Lancaster University,
Bailrigg, Lancaster, LA1 4YQ, United Kingdom.*

***For correspondence:**

E-mail: m.r.roberts@lancaster.ac.uk

Phone: +44 1524 510210

Fax: +44 1524 525261

Abstract

The function in plants of the non-protein amino acid, γ -aminobutyric acid (GABA) is poorly understood. In this study, we show that GABA down-regulates the expression of a large subset of 14-3-3 gene family members in *Arabidopsis thaliana* seedlings in a calcium, ethylene and abscisic acid-dependent manner. Gene expression is not affected when seedlings are supplied with glutamate, a precursor of GABA. The repression of 14-3-3 gene expression by GABA is dependent on functional ethylene and abscisic acid signalling pathways, since the response is lost in the *etr1-1*, *abi1-1* and *abi2-1* mutants. Calcium measurements show that in contrast to glutamate, GABA does not elicit a cytoplasmic calcium elevation, suggesting that the GABA response is unlikely to be mediated by glutamate receptors, as has been suggested previously. We suggest that in addition to its role as a stress-related metabolite, GABA may regulate gene expression in *Arabidopsis*, including members of the 14-3-3 gene family.

Keywords: *14-3-3 protein; GABA; Arabidopsis thaliana*

Introduction

Although nitrogen is thought to act as a signal in plants, very little is known about how this occurs (Lea & Miñin 2003). In animals and yeast, amino acids and their derivatives are thought to be important in nutrient signalling (Hyde, Taylor & Hundal 2003). In addition, the amino acids glutamate, glycine, glutamine and γ -aminobutyric acid (GABA), have well-established roles as neurotransmitters in the mammalian central nervous system (Reimer et al. 2001). It has been suggested that some of these nitrogen-containing molecules, including glutamate, glycine and GABA may also have potential signalling functions in plants (Lam et al. 1998; Bouché, Lacombe & Fromm 2003; Dubos et al. 2003).

GABA is a non-protein amino acid that comprises a significant fraction of the free amino acid pool in plant cells. GABA was first identified in plants over 50 years ago, but its significance remained obscure until the discovery that GABA levels are rapidly elevated in response to a range of abiotic and biotic stresses, including pathogen infection, wounding, cold and heat shock, drought and hypoxia. Subsequently, a number of possible roles in plants for GABA and the GABA shunt, the pathway in which it is synthesised, have been proposed (Shelp, Brown & McLean 1999; Bouché & Fromm 2004). These range from regulation of cytosolic pH and acting as a buffering mechanism in carbon and nitrogen metabolism, to protection against oxidative stress and defence against nematodes and herbivorous insects. Because of the rapid increases in GABA concentration during these stresses, GABA has also been suggested as a likely plant signalling molecule by several authors, (Kinnersley & Turano 2000; Bouché & Fromm 2004), though strong experimental evidence for this is lacking. In plant development, roles for GABA have been suggested in the control of pollen tube growth and guidance (Palanivelu et al. 2003), and senescence (Masclaux et al. 2000; Ansari, Lee & Chen 2005). The application of exogenous GABA has been shown to affect the expression of

ACC synthase in sunflower (Kathiresan et al. 1997) and nitrate uptake in *Brassica napus* (Beuve et al. 2004). However, the question of whether GABA plays a genuine signalling role in plants remains to be addressed.

In animals, GABA operates through ionotropic GABA_A and GABA_C receptors (Burt 2003), and through G-protein coupled metabotropic GABA_B receptors (Billinton et al. 2001).

Although homologues of GABA receptors are not present in plant genomes, there are reasons to suspect that GABA could interact with plant glutamate receptors (GLRs). A family of GLRs has been found in plants with high sequence similarity to animal GLRs (Lam et al. 1998; Lacombe et al. 2001; Turano et al. 2001) and they possess a regulatory domain that shares structural homology with mammalian GABA_B receptors (Turano et al. 2001). Plant GLRs are generally assumed to be involved in glutamate signalling, though there is evidence that they can also accept glycine as a ligand (Dubos et al. 2003). Glutamate signalling via plant GLRs has been related to calcium signalling and calcium utilization, since they appear to function as ligand-gated calcium channels (Kim et al. 2001; Dubos et al. 2003; Demidchik, Essah & Tester 2004).

Over recent years, 14-3-3 proteins have emerged as important players in the regulation of carbon and nitrogen metabolism in plants. 14-3-3 proteins are ubiquitous regulatory proteins that function by interacting directly with a wide range of target proteins, usually in a phosphorylation-dependent manner. They are involved in the regulation of various processes in plants, including N and C metabolism, in which they regulate enzymes such as nitrate reductase, glutamine synthetase, starch synthase III and glyceraldehyde-3-phosphate dehydrogenase (Huber, MacKintosh & Kaiser 2002; Comparot, Lingiah & Martin 2003). As well as regulating several nitrogen metabolic enzymes, 14-3-3 proteins have also been shown

to interact with proteins involved in metabolic signalling, such as calcium-dependent protein kinases (Camoni, Harper & Palmgren 1998; Moorhead et al. 1999) and SnRK1-related protein kinases (Ikeda et al. 2000) that phosphorylate 14-3-3 targets like nitrate reductase. 14-3-3 gene family members are differentially regulated during a range of stress responses (Roberts & Bowles 1999; Roberts, Salinas & Collinge 2002). We were therefore interested to determine whether 14-3-3 proteins themselves might be targets for regulation by nitrogen metabolic signalling. Here we report that the transcriptional regulation of Arabidopsis 14-3-3 genes is affected by GABA, but not other N sources tested. In the presence of high external calcium, GABA substantially represses transcription of most of the 14-3-3 genes expressed in Arabidopsis seedlings. Furthermore, we show that this effect is dependent on ethylene and ABA, suggesting the involvement of several signalling pathways in the response.

Materials and Methods

Plant materials

Wild-type and mutant plants used in this study were all derived from the Columbia (Col-0) ecotype of *Arabidopsis thaliana*. Plants were grown on Murashige and Skoog (MS) macro- and micronutrients using 1/10 nitrogen (2 mM NH_4^+ , 4 mM NO_3^-) and 3% (w/v) sucrose with the addition of other compounds as follows: 4 mM nitrate; 10 mM aspartate, glutamate or GABA; 50 μM 1-aminocyclopropane-1-carboxylic acid; 2 or 22 mM CaCl_2 . Shoots for gene expression analysis were collected from seven-day-old seedlings grown at 22-24°C in long days (16 h light-8 h dark) at a photosynthetically active irradiance of 80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$.

Measurement of gene expression by reverse transcription-polymerase chain reaction

Gene-specific primers corresponding to each of the 15 Arabidopsis 14-3-3 (*Grf*) genes were designed to enable RT-PCR analysis of gene expression. The specificity of the primers was tested by sequencing the PCR products. The 15 primer sets were used to perform PCR with Taq DNA polymerase (Red Taq, Sigma-Aldrich Company Ltd., UK) using first strand cDNA templates produced with Thermoscript RT (Invitrogen, UK) from 10 µg total RNA. All 15 sets of primers were designed to span intron sequences, allowing us to distinguish signals resulting from genomic DNA contamination. All primers were used at a concentration of 0.3 µM. Thermal cycling times and temperatures were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension period of 72°C for 10 min. Because of the difference in primer efficiencies, we cannot compare the expression levels between different 14-3-3 genes. We therefore assessed the relative difference in expression levels across different treatments for each *Grf* gene, respectively. The *Grf1* and *Grf2* genes served as good internal controls for cDNA synthesis, since the amplification of RT-PCR products from these two genes remained constant in all experiments.

***In vivo* reconstitution of aequorin and Ca²⁺ measurements**

Calcium measurements were carried out essentially as described by Knight, Trewavas & Knight (1996). Arabidopsis seedlings expressing aequorin (line RLD1.1; Polisensky & Braam 1996) were grown on MS agar plates with full strength nitrogen plus 22 mM CaCl₂. Six-day-old seedlings were transferred into water and coelenterazine solution (Lux Biotechnology, Edinburgh, UK; final concentration 4 µM, 1% (v/v) methanol) was added. Reconstitution of aequorin was allowed to proceed for a minimum of 16 h at 21°C in the dark. Luminescence was measured using a digital chemiluminometer with discriminator and cooled housing unit

(Electron Tubes, Ruislip, UK). After aequorin reconstitution, RLD1.1 seedlings were placed individually into cuvettes containing 0.5 mL water and inserted into the luminometer chamber. Following a resting period of 3 min to recover from the touch response, luminescence counts were recorded at 1 second intervals. After 100 s of counting, 1 mL of 1.5 mM glutamate or GABA solution was added from a syringe through a luminometer port and measurements continued over the indicated time periods. After 200 s of counting, 1 mL cold water was added. The remaining aequorin was discharged by addition of 2 mL 0.9 M CaCl₂ solution in 10% ethanol, and counts recorded for a further 2 min. The luminescence counts obtained were calibrated by applying the equation: $pCa = 0.332588(-\log k) + 5.55593$, where $k = \text{luminescence counts s}^{-1} / \text{total luminescence counts remaining}$ (Knight et al. 1996).

Western blot analysis

Crude protein extracts from shoots (20 µg total protein per sample) were separated on NuPAGE™ 4-12% Bis-Tris gels (Invitrogen). For western analysis, proteins were transferred onto PVDF membranes and immunodetection was performed using a 500-fold dilution of a rabbit antiserum containing polyclonal antibodies raised against recombinant tomato 14-3-3 isoform TFT4. This antibody recognises non-epsilon forms of 14-3-3 (A. P. Brown & M. R. Roberts, unpublished).

Results

GABA represses 14-3-3 gene expression in Arabidopsis seedlings

Fifteen genes encoding 14-3-3 isoforms have been identified in Arabidopsis and are named *Grf1* to *Grf15* (Rosenquist et al. 2001), of which two (*Grf14* and *Grf15*) are likely pseudogenes. Using reverse transcription PCR (RT-PCR), we routinely identified the transcripts of nine 14-3-3 genes, *Grf1-Grf9*, in young seedlings under the conditions studied. In order to investigate the effect of different nutrients on 14-3-3 gene expression, we used RT-PCR to assay 14-3-3 mRNA levels in seedlings grown at both normal (2 mM) and high (22 mM) calcium, in the presence or absence of different nitrogen compounds. We found that GABA, but not other nitrogen sources, including glutamate (Fig. 1), aspartate, and nitrate (data not shown), caused a significant reduction in transcript levels of the majority of the expressed 14-3-3 genes when plants were grown on media containing high calcium (Fig. 1). At 2 mM calcium, neither glutamate nor GABA significantly affected 14-3-3 gene expression, with the exception of the repression of *Grf8* by GABA (Fig. 1). The *Grf1* and *Grf2* genes were not appreciably affected by GABA, and serve as good internal controls. These results show that calcium and GABA interact to affect the expression of a large sub-set of 14-3-3 genes. Neither GABA nor high calcium had any visible effect on post-germinative growth compared to controls, either alone or in combination.

In order to see whether the effect of GABA on 14-3-3 mRNA levels was reflected at the protein level, we performed western blots using an antibody raised against a tomato 14-3-3 isoform, TFT4. This antibody recognises the majority of, though not all, 14-3-3 isoforms in plants (A.P. Brown and M.R. Roberts, unpublished data). We found that 14-3-3 protein levels were decreased by around three- to four-fold in response to GABA at high calcium (Fig. 2).

These results suggest a reduction in total cellular 14-3-3 protein levels mediated at the transcriptional level.

GABA responses require intact ethylene and ABA signalling pathways

Abscisic acid (ABA) and ethylene signalling are important in responses to stress and in the regulation of primary metabolism in *Arabidopsis* seedlings (Gazzarrini & McCourt 2001). Since GABA might be involved in responses to stress and/or nutrients, we were interested to test whether these signalling pathways might also be involved in the response of 14-3-3 genes to GABA. To test the involvement of ethylene, we first grew seedlings on media supplemented with 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor for ethylene biosynthesis. RT-PCR analysis showed that in all cases, the repressive effect of GABA on 14-3-3 gene expression was counteracted by the presence of ACC. Representative examples are shown in Fig. 3. We then went on to investigate the role of ethylene in the response to GABA using a genetic approach. Seedlings of the ethylene-insensitive mutant, *etr1-1*, were grown on media at normal or high calcium and in the presence or absence of glutamate or GABA. As in the wild-type Col-0 background, the expression of each 14-3-3 gene was similar at each condition in *etr1-1* seedlings grown on 2 mM calcium (data not shown). At high calcium, however, we found that the pattern of 14-3-3 gene expression differed from that in Col-0. Firstly, the expression of the majority of 14-3-3 genes, though not *Grf2*, *Grf6* or *Grf8*, was reduced relative to Col-0 in the absence of supplementary nitrogen. These patterns of expression are illustrated by representative examples in Fig. 3. Secondly, we found that for all genes, the addition of glutamate or GABA resulted in expression levels similar to those found in Col-0 plants grown in the absence of GABA (Fig. 3). These data suggest that expression of

some 14-3-3 genes is down-regulated by high calcium in *etr1-1* mutant plants, but that the repressive effect of GABA is lost.

We also used mutants to determine whether there might be a role for ABA in the regulation of 14-3-3 genes. We found that the repression of 14-3-3 expression by GABA at high calcium was completely lost in the ABA-insensitive mutants, *abi1-1* and *abi2-1*. Representative examples are included in Fig. 3. No differences in 14-3-3 expression between Col-0 and *abi1-1* or *abi2-1* were observed under any other conditions (not shown). This result indicates a central role for ABA signalling in the response to GABA.

Calcium signals elicited by GABA and glutamate

Previous results have shown that glutamate stimulates transient elevations in cytoplasmic calcium concentration ($[Ca^{2+}]_{cyt}$) in plant cells (Dennison & Spalding 2000; Dubos et al. 2003). Since it has been suggested that GABA might act via GLRs in plants (Bouché et al. 2003), we were interested to test whether GABA is also able to stimulate $[Ca^{2+}]_{cyt}$ elevations in Arabidopsis seedlings. As shown in Fig. 4A, 1 mM GABA did not induce a $[Ca^{2+}]_{cyt}$ elevation. In contrast, when 1 mM glutamate was supplied to the seedlings, a clear transient elevation of cytosolic calcium was detected (Fig. 4B). A cold treatment was used as a control to show that the seedlings tested were similarly competent for a cold-induced $[Ca^{2+}]_{cyt}$ elevation. We also tested whether GABA was able to alter the calcium signal elicited by glutamate, but no difference was found when glutamate was applied alone or in combination with GABA (data not shown).

Discussion

GABA regulates 14-3-3 gene expression in Arabidopsis seedlings.

Since 14-3-3 proteins regulate several aspects N metabolism in plants, we were interested to determine whether the 14-3-3s themselves might be subject to regulation by N metabolites. We therefore examined the expression of all members of the 14-3-3 gene family in Arabidopsis seedlings grown with different forms of supplementary N. Amongst the different organic (amino acids) and inorganic (nitrate) N compounds that were initially tested, only GABA showed a significant effect. We found that in the presence of high external calcium concentrations, GABA down-regulates a large sub-set of 14-3-3 genes via a pathway that requires proteins involved in ethylene and ABA signalling. Since GABA is derived from glutamate metabolism, we paid particular attention to the effect of glutamate in comparison with GABA in all experiments. Glutamate has been shown to regulate gene expression of some metabolic enzymes such as nitrate reductase in tobacco (Stitt et al. 2002) and glutamine synthetase in Arabidopsis (Oliveira & Coruzzi 1999). However, our results showed that the transcriptional response of 14-3-3 genes is specific to GABA. This also argues against the possibility that the observed changes in 14-3-3 gene expression might occur as a consequence of GABA acting solely as a nutrient. All control plants were grown on MS medium containing 2 mM ammonium and 4 mM nitrate, which represents sufficient N for growth of Arabidopsis seedlings. More importantly, of several different utilisable sources of N, GABA was the only compound tested which resulted in a change in 14-3-3 gene expression. This argues against a general effect of GABA acting directly as a nutrient, or by altering C:N balance in our system. Instead, our data suggest a model in which GABA, directly or indirectly, generates a specific response that includes the down-regulation of 14-3-3 genes. Although the concentration of GABA used in our experiments appears relatively high, GABA

has been estimated to reach levels of up to 2 $\mu\text{mol g}^{-1}$ fresh weight in extracts from plants undergoing abiotic stress responses, and accumulates to levels approaching 40 mM in the media of osmotically-stressed cultured cells (Shelp et al. 1999 and references therein). *Arabidopsis* can grow on medium containing GABA as a sole source of nitrogen (Breitkreuz et al. 1999), and recent work has identified a high affinity GABA transporter, confirming that plants have a mechanism to take up GABA from the extracellular medium (Meyer et al. 2006). In our system, the decrease of 14-3-3 transcript levels in response to GABA was accompanied by a decrease in the 14-3-3 protein level, suggesting that the GABA effect is likely to have a significant physiological impact.

Interactions between GABA, high external calcium, and ethylene and ABA signalling pathways

A detailed examination of the mechanism of the GABA response showed that proteins of ethylene signalling (ETR1) and ABA signalling (ABI1 and ABI2) are required for the repression of 14-3-3 genes. Firstly, we found that the effect of GABA on 14-3-3 regulation depends on a functional ethylene signalling pathway. When ethylene is constitutively present (provided in the form of its precursor, ACC), GABA no longer has any effect on 14-3-3 expression. Paradoxically, when ethylene perception is inhibited by the *etr1-1* mutation, the ability of GABA to down-regulate 14-3-3 gene expression is also lost. This suggests an optimum level of ethylene signalling for 14-3-3 gene expression, whereby a loss or excess of ethylene signalling activity results in a failure to respond to GABA. The effect of the *etr1-1* mutation on basal 14-3-3 expression levels at high calcium could be the result of a general defect in N metabolism, since expression is restored to wild-type levels by the addition of either glutamate or GABA.

The role of exogenous calcium in the regulation of 14-3-3 gene expression is complex. For some plants, high rhizospheric calcium can have a negative impact on plant growth (Lee 1999), though experiments with *Arabidopsis* grown on agar media suggested no significant toxicity at concentrations up to 30 mM (Chan et al. 2003). In our experiments, high calcium concentration has no effect on 14-3-3 transcripts in wild-type plants, and GABA affects gene expression only in the presence of elevated calcium (with the exception of the repression of *Grf8*, which also occurs at normal calcium levels). In contrast, in *etr1-1*, exogenous calcium levels have a significant effect on 14-3-3 expression even in the absence of GABA. These data suggest a complex relationship between ethylene signalling, calcium and N nutrition. Currently, the physiological significance of our results is not clear. GABA is unlikely to be acting on a nutrient signalling pathway operating under normal physiological conditions, since the response requires high external calcium concentrations. However, the affect of GABA plus high calcium on 14-3-3 gene expression is not insignificant. Perhaps the simplest explanation of our data would be that calcium and GABA interact to signal specific forms of environmental stress. This idea is consistent with the requirement for ethylene and ABA signalling downstream of calcium and GABA.

Multiple roles for GABA in plant tissues

Many mechanisms have been suggested by which GABA may function in plant tissues. Its metabolism through the GABA shunt is thought to be involved in protection against oxidative stress (Coleman et al. 2001; Bouché et al. 2003) and pH regulation (Shelp et al. 1999). GABA itself has been proposed to function as a buffer for glutamate and other amino acids, and as a potential compatible osmolyte in protection against osmotic stress (Shelp et al. 1999).

Because of its role as a neurotransmitter in animals, GABA is also believed to act as a neurotoxin when ingested by nematodes and herbivorous insects (McLean et al. 2003; MacGregor et al. 2003). GABA has also been identified as a possible signalling molecule involved in pollen tube growth and guidance (Palanivelu et al. 2003) and in the regulation of nitrate uptake (Beuve et al. 2004). Our data now suggest that GABA may also influence gene expression in plants, perhaps as a component of stress responses, with 14-3-3 proteins as one important target.

Acknowledgements

We thank Liz Neame for assistance with 14-3-3 gene expression analyses, Dr Dawn Worrall for assistance with the calcium measurements and Professors Peter Lea and Brian Forde for critical reading of the manuscript. ML was supported by a grant from the UK Biotechnology and Biological Sciences Research Council. MRR was supported by a University Research Fellowship from the Royal Society.

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Figures

Figure 1. 14-3-3 gene expression is repressed by the presence of GABA.

RT-PCR analysis of 14-3-3 (*Grf*) gene expression in control plants and plants grown in the presence of supplementary glutamate (GLU) or GABA. Media contained either normal (2 mM) or high (22 mM) calcium. The figure shows representative examples of PCR products on ethidium bromide-stained gels.

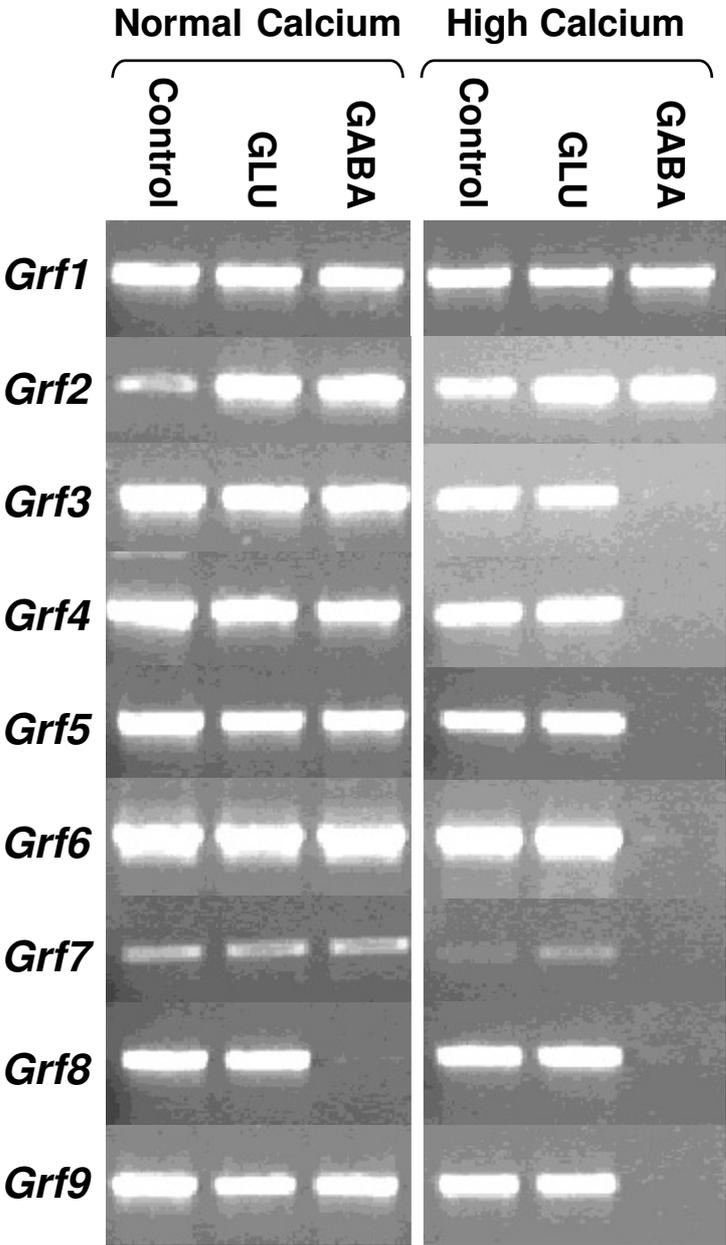


Figure 2. 14-3-3 protein levels are reduced by the presence of GABA

Western blot analysis of 14-3-3 proteins from seedlings grown in the absence of organic nitrogen (-) or in the presence of GABA (+) at high calcium, probed using anti-TFT4 antibodies. Total protein loading was visualised by Coomassie blue staining of the PVDF membrane after transfer. A representative portion of the membrane is shown.

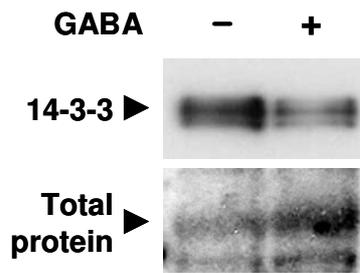


Figure 3. Interactions between ethylene and ABA signalling and 14-3-3 expression in response to GABA.

mRNA RT-PCR analysis of 14-3-3 (*Grf*) gene expression in control plants and plants grown at high calcium in the presence of supplementary ACC, glutamate (GLU) or GABA. Plants were either the wild-type or *etr1-1*, *abi1-1* and *abi2-1* mutants in the Col-0 background. The figure shows representative examples of PCR products on ethidium bromide-stained gels.

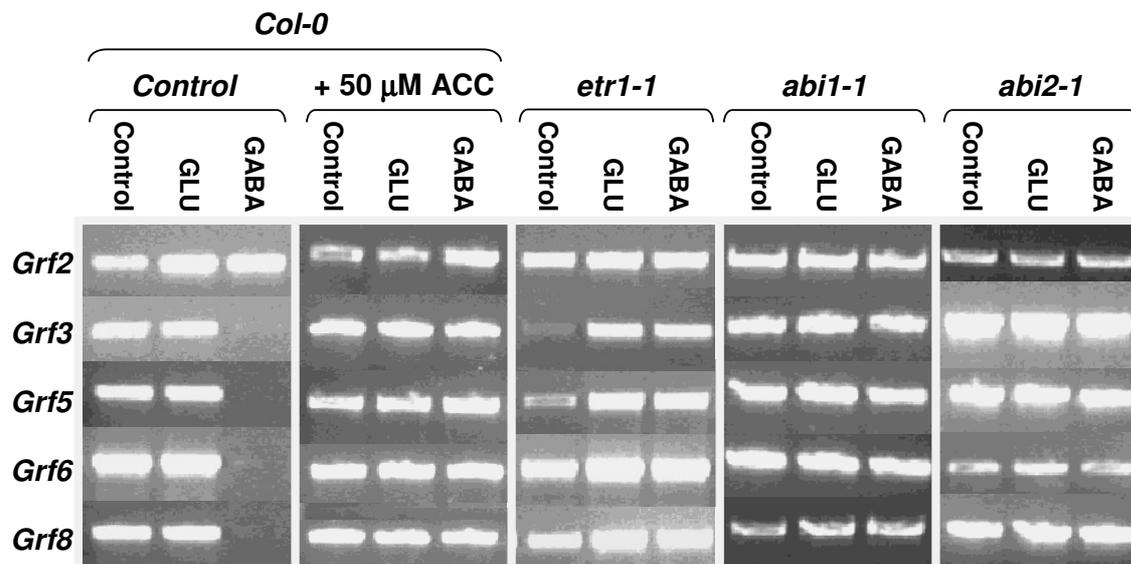


Figure 4. Effects of GABA and glutamate on $[Ca^{2+}]_{cyt}$ in Arabidopsis seedlings.

Calcium-dependent luminescence from aequorin was measured using a photon-counting luminometer. Time course of GABA (**a**) and glutamate (**b**)-induced luminescence for a typical single wild-type seedling. Nitrogen compounds (1 mM GABA or 1 mM glutamate (GLU)) and cold water treatment, used as a positive control, were applied at the times indicated.

