Regulation of *Arabidopsis thaliana* 14-3-3 gene expression by γ-aminobutyric acid

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**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 (ABA)-dependent manner. Gene expression is not affected when seedlings are supplied with glutamate (GLU), a precursor of GABA. The repression of 14-3-3 gene expression by GABA is dependent on functional ethylene and ABA signalling pathways, because the response is lost in the *etr1-1, abil-1* and *abi2-1* mutants. Calcium measurements show that in contrast to GLU, GABA does not elicit a cytoplasmic calcium elevation, suggesting that the GABA response is unlikely to be mediated by GLU receptors (GLRs), as has been suggested previously. We suggest that in addition to its role as a stress-related metabolite, GABA may regulate gene expression in *A. thaliana*, including members of the 14-3-3 gene family.

Key-words: GABA, amino acids, calcium.

**INTRODUCTION**

Although nitrogen is thought to act as a signal in plants, very little is known about how this occurs (Lea & Miflin 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 (GLU), 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 GLU, 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 synthesized, 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), although a 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 (Palaniyvelu 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 1-aminocyclopropane-1-carboxylic acid (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<sub>A</sub> and GABA<sub>C</sub> receptors (Burt 2003), and through G-protein coupled metabotropic GABA<sub>B</sub> receptors (Billington et al. 2001). Although homologs of GABA receptors are not present in plant genomes, there are reasons to suspect that GABA could interact with plant GLU 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<sub>B</sub> receptors (Turano et al. 2001). Plant GLRs are generally assumed to be involved in GLU signalling, although there is evidence that they can also accept glycine as a ligand (Dubos et al. 2003). GLU signalling via plant GLRs has been related to calcium signalling and calcium utilization, because 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 nitrogen and carbon 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 (Camon, Harper & Palmgren 1998; Moorhead et al. 1999) and sucrose non-fermenting-1-related protein kinase 1 (SnRK1)-related protein kinases (Ikeda et al. 2000) that phosphorylate 14-3-3 targets like nitrate reductase. The 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 thaliana 14-3-3 genes is affected by GABA, but not other nitrogen sources tested. In the presence of high external calcium, GABA substantially represses the transcription of most of the 14-3-3 genes expressed in A. thaliana seedlings. Furthermore, we show that this effect is dependent on ethylene and abscisic acid (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 A. thaliana. The plants were grown on Murashige and Skoog (MS) macro- and micronutrients using 1/10 nitrogen (2 mM NH₄NO₃, 4 mM NO₃⁻) and 3% (w/v) sucrose with the addition of other compounds as follows: 4 mM nitrate; 10 mM aspartate, GLU or GABA; 50 μM ACC; 2 or 22 mM CaCl₂. Shoots for gene expression analysis were collected from 7-day-old seedlings grown at 22–24 °C in long days (16 h light–8 h dark) at a photosynthetically active irradiance of 80 μmol quanta m⁻² s⁻¹.

Measurement of gene expression by reverse transcription (RT)–PCR

Gene-specific primers corresponding to each of the 15 A. thaliana 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, Gillingham, UK) using first-strand cDNA templates produced with Thermoscript RT (Invitrogen, Paisley, 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, because the amplification of RT–PCR products from these two genes remained constant in all the experiments.

In vivo reconstitution of aequorin and calcium measurements

Calcium measurements were carried out essentially as described by Knight, Trewavas & Knight (1996). Arabidopsis thaliana 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, Scotland; 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, England). 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 s intervals. After 100 s of counting, 1 mL of 1.5 mM GLU 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 the addition of 2 mL of 0.9 M CaCl₂ solution in 10% ethanol, and counts were recorded for a further 2 min. The luminescence counts obtained were calibrated by applying the following equation: pCa = 0.332588(−log k) + 5.5593, where k = luminescence counts s⁻¹/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(2-hydroxyethyl)amino]tri(hydroxyethyl)methane (bis-Tris) gels (Invitrogen). For Western blot analysis, proteins were transferred onto polyvinylidene difluoride (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, tomato fourteen-three-four (TFT4). This antibody recognizes the non-epsilon forms of 14-3-3 (Brown & Roberts, unpublished results).
RESULTS

GABA represses 14-3-3 gene expression in A. thaliana seedlings

Fifteen genes encoding 14-3-3 isoforms have been identified in A. thaliana and are named Grf1 to Grf15 (Rosenquist et al. 2001), of which two Grf14 and Grf15 are likely pseudogenes. Using RT–PCR, we routinely identified the transcripts of nine 14-3-3 genes, Grf1 to Grf9, in young seedlings under the conditions studied. 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 GLU (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 GLU 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 subset 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.

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 recognizes the majority of, although not all, 14-3-3 isoforms in plants (Brown & Roberts, unpublished results). We found that 14-3-3 protein levels were decreased by around three- to fourfold 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

ABA and ethylene signalling is important in responses to stress and in the regulation of primary metabolism in A. thaliana seedlings (Gazzarrini & McCourt 2001). Because 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 ACC, a precursor for ethylene biosynthesis. RT–PCR analysis showed that in all cases, the repressive effect

Figure 1. The 14-3-3 gene expression is repressed by the presence of γ-aminobutyric acid (GABA). Reverse transcription (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. The 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. The 14-3-3 protein levels are reduced by the presence of γ-aminobutyric acid (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-tomato fourteen-three- four (TFT4) antibodies. The total protein loading was visualized by Coomassie blue staining of the polyvinylidene difluoride (PVDF) membrane after transfer. A representative portion of the membrane is shown.
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 GLU 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, although 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 GLU 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 GLU

Previous results have shown that GLU stimulates transient elevations in cytoplasmic calcium concentration ([Ca^{2+}]_{cyt}) in plant cells (Dennison & Spalding 2000; Dubos et al. 2003). Because 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 A. thaliana seedlings. As shown in Fig. 4a, 1 mM GABA did not induce a [Ca^{2+}]_{cyt} elevation. In contrast, when 1 mM GLU 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 GLU, but no difference was found when GLU was applied alone or in combination with GABA (data not shown).

DISCUSSION

GABA regulates 14-3-3 gene expression in A. thaliana seedlings

Because 14-3-3 proteins regulate several aspects of nitrogen metabolism in plants, we were interested to determine whether the 14-3-3s themselves might be subject to regulation by nitrogen metabolites. We therefore examined the expression of all members of the 14-3-3 gene family in A. thaliana seedlings grown with different forms of supplementary nitrogen. Among the different organic (amino
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 an 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 nitrogen metabolism, because expression is restored to wild-type levels by the addition of either GLU or GABA.

The role of exogenous calcium in the regulation of 14-3-3 gene expression is complex. For some plants, high atmospheric calcium can have a negative impact on plant growth (Lee 1999), although experiments with *A. thaliana* 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 expression 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.
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 GLU 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 (MacGregor et al., 2003; McLean 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.

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