Plant Science 245 (2016) 25–34

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Gamma-aminobutyric acid depletion affects stomata closure and drought tolerance of *Arabidopsis thaliana*

Dereje Worku Mekonnen[a,*], Ulf-Ingo Flügge[a], Frank Ludewig[a,b]

[a] Botanical Institute II, Cologne Biocenter, University of Cologne, Zülpicher Str. 47b, 50674 Cologne, Germany
[b] Division of Biochemistry, Department Biology, University Erlangen-Nuremberg, Staudtstr. 5, 91058 Erlangen, Germany

ARTICLE INFO

Article history:
Received 14 October 2015
Received in revised form 13 January 2016
Accepted 20 January 2016
Available online 23 January 2016

Keywords:
GABA
Glutamate decarboxylase
Stomatal conductance
Stomata closure
POP2

ABSTRACT

A rapid accumulation of γ-aminobutyric acid (GABA) during biotic and abiotic stresses is well documented. However, the specificity of the response and the primary role of GABA under such stress conditions are hardly understood. To address these questions, we investigated the response of the GABA-depleted *gad1/2* mutant to drought stress. GABA is primarily synthesized from the decarboxylation of glutamate by glutamate decarboxylase (GAD) which exists in five copies in the genome of *Arabidopsis thaliana*. However, only *GAD1* and *GAD2* are abundantly expressed, and knockout of these two copies dramatically reduced the GABA content. Phenotypic analysis revealed a reduced shoot growth of the *gad1/2* mutant. Furthermore, the *gad1/2* mutant was wilted earlier than the wild type following a prolonged drought stress treatment. The early-wilting phenotype was due to an increase in stomata aperture and a defect in stomata closure. The increase in stomata aperture contributed to higher stomatal conductance. The drought oversensitive phenotype of the *gad1/2* mutant was reversed by functional complementation that increases GABA level in leaves. The functionally complemented *gad1/2 x pop2* triple mutant contained more GABA than the wild type. Our findings suggest that GABA accumulation during drought is a stress-specific response and its accumulation induces the regulation of stomatal opening thereby prevents loss of water.

1. Introduction

γ-Aminobutyric acid (GABA), a non-protein amino acid, has first been discovered in potato tubers half a century ago [1]. In Arabidopsis, GABA is primarily produced from the decarboxylation of glutamate in the cytosol. The pathway that involves the synthesis and breakdown of GABA is called GABA shunt [2–4]. The GABA shunt is conserved from bacteria to higher organisms like plants and animals [6]. In plants and mammals, GABA synthesis occurs in the cytosol and its degradation takes place in mitochondria [5,7]. In Arabidopsis, the GABA shunt consists of three enzymes, i.e., cytosolic glutamate decarboxylase (GAD), mitochondrial GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH). Unlike GAD, which is encoded by five copies in the Arabidopsis genome [8], GABA-T [9] and SSADH [10] are encoded by single copy genes.

The five *GAD* genes share significant sequence similarity and show organ-specific expressions [11]. For example, at amino acid level GAD1 and GAD2 share 82% sequence identity [12]. *GAD1* (At5g17330) is mainly expressed in roots, whereas *GAD2* (At1g65960) transcripts are detectable in almost all organs [12,13]. *GAD3* (At2g02000) and *GAD4* (At2g02010) are weakly expressed in shoots and roots under normal growth conditions. *GAD4* transcript was up-regulated in shoots when plants were exposed to salt stress conditions [14]. *GAD5* (At3g17760) is mainly expressed in the male gamete [15]. GAD catalyzes the synthesis of GABA from glutamate in the cytosol, and its activity is enhanced at acidic pH [8,16]. The C-termini of AtGAD1 and AtGAD2 contain Ca^{2+}/CaM binding domains, which upon binding of a Ca^{2+}/CaM complex stimulate the activity of the protein [17]. The C-terminus of rice GAD was shown to have an auto-inhibitory role, as the truncated version promoted the over-accumulation of GABA [17]. Furthermore, Baum et al. [18] reported the importance of the CaM binding domain of GAD for the regulation of GABA metabolism in petunia plants. Tobacco plants over-expressing petunia GAD lacking the C-terminus exhibited abnormal growth which was associated with elevated GABA and reduced glutamate levels.

GABA accumulates rapidly in response to biotic and abiotic stresses [19,20]. Roles of GABA in maintaining C/N balance, regulating cytosolic pH, scavenging of reactive oxygen species, etc. have previously been discussed [21]. The involvement of GABA in

* Corresponding author at: Rudolf Breitscheid Str. 8, 51067 Cologne, Germany.
E-mail addresses: Derejeworku1@yahoo.com (D.W. Mekonnen), ui.fluegge@uni-koeln.de (U.-I. Flügge), Frank.ludewig@fau.de (F. Ludewig).

http://dx.doi.org/10.1016/j.plantsci.2016.01.005
0168-9452/

reproduction has also been reported. The Arabidopsis *gaba-t* (*pop2*) mutant, that accumulates GABA in shoots and flowers, exhibited a defect in pollen tube elongation as shown by *in vitro* and *in vivo* experiments [22–24]. The effect of GABA on vegetative growth by modifying the gene expression pattern [23] demonstrated the wide range of roles GABA and possibly its derivatives could play in plant growth and development. Very recently, the physical interaction of GABA and the ALMT1 protein, thereby affecting plant growth, has been reported [23,25]. The authors described the interaction to be characteristic for ALMT family proteins, and also speculatively suggested this interaction to affect stomatal movement. ALMT6 and ALMT12 proteins, localized in the vacuolar and plasma membrane, respectively, are involved in the regulation of stomatal movement by allowing the influx and efflux of malate [26,27]. The work of Meyer et al. [27] suggested the existence of a cofactor (cytosolic or vacuolar) that binds and modulates the activity of ALMT6 protein, as the removal of Ca^{2+} from the already activated protein did not change the activity.

Despite the rapid accumulation of GABA during stresses, the specificity of the response and the specific role of GABA under these conditions are still elusive. Furthermore, GABA-depleted mutants have not been extensively used to study GABA functions. To date only the effect of excess GABA, either by feeding GABA exogenously or by using mutant lines with elevated GABA, on plants has been reported. Recently, the GABA-depleted Arabidopsis *gad1/2* double mutant and the GABA-enriched *gad1/2 x pop2-5* triple mutant have been used to study the effect of GABA on the feeding habit of the insect larvae *Spodoptera littoralis* [28]. The result confirmed that the growth performance of insect larvae may be reduced by feeding on plants with elevated GABA levels [28,29].

In this study, we used the GABA-depleted Arabidopsis *gad1/2* and GABA-rich *gad1/2 x pop2-5* mutants [28] to investigate the specificity of the GABA response, and suggest the role of GABA in drought stress response. The GABA-depleted *gad1/2* mutant wilted earlier than the wild type following a prolonged drought stress. The early wilting phenotype was correlated with an increase in stomatal conductance. This increase in stomatal conductance was attributed to an increase in stomata aperture. The wilty phenotype was reversed to that of the wild type by functional complementation, which increases the GABA level in shoots. Our findings suggest that GABA accumulation is a specific response. Furthermore, we propose that GABA acts as a signaling molecule during drought stress by binding to GABA receptors (ALMT proteins) and also modulates the activity of H^+-ATPase via 14-3-3 proteins, thereby regulating stomatal movement.

2. Materials and methods

2.1. Plant materials

F1 seeds of *gad1* (At5g17330; SALK_017810), *gad2* (At1g65960; GK_474E05) and *pop2-5* (At3g22200; GK_157D10) were obtained from the respective stock centers [28]. Screening of F2 plants homozygous for the knockout of the respective genes was carried out as described before [28]. The generation and isolation of *gad1/2* double mutants and *gad1/2 x pop2-5* triple mutants was described in a previous paper [28].

2.2. Growth conditions

2.2.1. Greenhouse

Seeds of wild type and *gad1*, *gad2, gad1/2, gad1/2 x pop2-5 and pop2-5* mutants were sown on soil and cold treated for two days in a 4° room before they were transferred to greenhouse for germination. At two-leaf stage (~10 days after sowing) the individual seedlings were transferred to pots of 6 cm in diameter filled with soil mix. Five pots from each genotype were placed completely randomized on two perforated trays and kept in the greenhouse with light/dark cycle of 16/8 h (supplemented with two fluorescence tubes overhead, Osram L18W/840) and relative humidity (RH) of approximately 40%. The plants were watered every three days by immersing the perforated trays into a bigger tray filled with water. For drought stress treatment, watering was withheld from three-week-old plants on one tray, and the control tray were continued to be watered every three days.

2.2.2. Growth chamber conditions

Seeds of wild type and *gad1/2* mutants were sown, cold treated for 2 days and germinated in the greenhouse. Approximately ten days after sowing, the seedlings were transferred to pots as described above. Five pots from both genotypes were placed completely randomized on two perforated trays. Then, the pots were transferred to short day conditions into a growth chamber (8/16 h light/dark cycle, 23 °C and 60–70% relative humidity). The watering of plants was carried out as described above by immersing in water-filled trays. For the drought stress treatment three-week-old plants on one tray were prevented from getting water (drought stress) and the other tray was continued to get water (control).

2.3. Water content analysis

To quantify the drought stress phenotype, the shoot water content was determined as follows: The entire shoots were harvested from five control and drought stress-treated wild-type and *gad1/2* plants. The fresh biomass of shoots was immediately weighed, wrapped with aluminum foil and oven dried at 80 °C for two days. The percent water content was calculated as shown below. The percent water content was plotted against the treatment duration.

$$\%WC = \frac{(FB\text{-}DB)}{FB} \times 100$$

WC: water content; FB: fresh biomass; DB: dry biomass

2.4. Analysis of stomatal conductance, stomata density and stomata aperture

Seeds of wild type, *gad1*, *gad2* and *gad1/2* were sown, cold treated and germinated on soil under greenhouse conditions. Approximately one week after germination, individual seedlings were transferred to small pots. Five pots from each genotype were placed on to two perforated trays in a completely randomized way. After three weeks of growth watering was stopped and the stomatal conductance of the abaxial and adaxial leaf sides was measured using a leaf porometer (Model SC-1, Decagon Devices, Inc.). The measurement was continued for six days. Five plants per genotype and three leaves per plant were measured every day. For stomata density analysis the third and fourth leaves were collected from wild type and *gad1/2* plants and chlorophyll was removed by ethanol treatment. Then, the leaves were placed on a slide and stomata were counted under a microscope. For each leaf three counts were made on three different microscopic fields. A total of 26 leaves were analyzed from each genotype. To determine stomata aperture, the abaxial side of leaves was polished with nail varnish. When it was dry, it was peeled off, placed on a slide and a picture was taken using a camera mounted on a microscope. From each leaf, pictures were taken from at least 20 different microscopic fields. From each field the aperture of three stomata was measured using the ImageJ software. A total of 6 leaves were analyzed from each genotype.