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Functioning of the γ-aminobutyrate pathway in wheat seedlings affected by osmotic stress

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Abstract

γ-Aminobutyrate (GABA) was the only amino acid out of three amino acid intermediates of GABA shunt that increased significantly after 28 h from the beginning of osmotic stress induced by 20 % polyethylene glycol 6000 in wheat seedlings. At the same time specific activities of glutamate decarboxylase (GAD) and GABA aminotransferase (GABA-T) two enzymes of GABA pathway did not change as compared with the control plants. The response of two GABA-T activities (with pyruvate or 2-oxoglutarate as amino acid acceptor) to aminooxyacetate, 3-chloro-L-alanine and *p*-hydroxymercuribenzoate prompted us to suggest that at least two isoforms of GABA-T showing different substrate specificity do exist in wheat leaves.

Additional key words: GABA aminotransferase, glutamate decarboxylase, GABA shunt, *Triticum aestivum*.

Introduction

γ-Aminobutyrate (GABA) is one of the most important neurotransmitters in mammalian central nervous system (Hampe *et al.* 2001). Its action consists in blocking transmission in nervous synapses (Hampe *et al.* 2001). This compound is also common in plants (Bown and Shelp 1997). Several-fold increase of GABA concentration was demonstrated as a response to various external stimuli like thermal shock, mechanical damage, oxygen shock, presence of phytohormones in plant cells (Rhodes *et al.* 1999) or pathogen infection (Solomon and Oliver 2001). GABA is formed as a result of decarboxylation of L-glutamate catalyzed by cytosolic glutamate decarboxylase (GAD, EC 4.1.1.15).

It is then transformed in the reaction of transamination involving mitochondrial GABA aminotransferase (GABA - T, EC 2.6.1.19.) and pyruvate or 2-oxoglutarate as amino group acceptors. One of the products of these transaminations succinic semialdehyde undergoes transformation to succinate in a reaction catalyzed by succinic semialdehyde dehydrogenase (EC 1.2.1.16) localized in mitochondria. These three reactions comprise the so-called GABA shunt (Shelp *et al.* 1999). Its role is to supply most of the carbon chains of L-glutamate in the form of succinate to the Krebs cycle (Shelp *et al.* 1999).

Various functions are proposed to the process of GABA synthesis. By consuming H^+ ions, the enzymatic decarboxylation of L-glutamate could stabilize cell pH (Bown and Shelp 1997). Some papers underline a potential role of GABA in regulating plant growth and development. By binding to proper receptors this amino acid could play regulatory functions (Chung *et al.* 1992, Chen *et al.* 1994, Kathiresan *et al.* 1997). According to Ramputh and Bown (1996), accumulation of GABA in leaves protects a plant against insect larvae attack. GABA, along with proline and quaternary amines, also participates as an osmolyte in plant adaptation to osmotic stress (Fischer *et al.* 1988, Breitkreutz *et al.* 1999, Schwacke *et al.* 1999, Rai 2002). Noteworthy, the stress is not always accompanied by GABA accumulation in plants (Shelp *et al.* 1999).

This research was aimed to find out if GABA accumulates in wheat seedlings in response to osmotic stress caused by 20 % polyethylene glycol 6000 (PEG

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Abbreviations: PMSF - phenylmethanesulfonyl fluoride; EDTA - ethylenediaminetetraacetic acid disodium salt; GABA - γ-aminobutyrate; GABA-T - γ-aminobutyrate aminotransferase; PLP - pyridoxal phosphate; GAD - glutamate decarboxylase; OPA - ortophtalaldehyde; WSD - water saturation deficit.

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6000). We also wanted to gain information about activity changes of GAD and GABA-T during stress. Additional goal was to elucidate whether GABA-T isoforms do exist in wheat leaves.

Materials and methods

Plants and treatments: Spring wheat leaves (*Triticum aestivum* L. cv. Jasna) was grown in hydroponics in distilled water (seeds were pre-treated with fungicide *Funaben T*) in cuvettes kept in a growing chamber at day/night temperature of 22/16 °C (15-h photoperiod). Photon flux density was 400 μmol m^{-2} s^{-1}. After 7 d, distilled water was replaced by medium containing 0.25 % commercial orchard fertilizer *Florovit*. After another 7 d plants in one cuvette were stressed by changing the medium for that containing additionally 20 % (m/v) of PEG 6000. In the other cuvette medium was replaced with the new one without stressing agent. 14-d old or older seedlings were collected for analyses by cutting them at a height of the first proper leaf at the fifth hour of a day (except for the first day of the experiment). Part of the sampled plant material was used to determine the water saturation deficit (WSD) (Miazek *et al.* 2001), which was adopted as a measure of dehydration of plant tissue:

$$WSD\ [\%] = [(W - W_{act})/(W - W_s)] \times 100$$

(W - mass of the sample after submersion in water overnight in the dark, W_{act} - mass of the sample when harvested, W_s - mass of the sample dried at a temperature of 100 °C for 24 h). The remaining plant material was frozen and stored at -80 °C.

Preparation of homogenate: Leaf tissue was homogenized for 90 s in *Ultra Turax T25* (*IKA Labortechnik*, Staufen, Germany) homogenizer in a 20 mM Tris-Gly buffer, pH 9.1, containing 1 mM EDTA, 0.5 mM pyridoxal phosphate (PLP), 10 mM 2-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride (PMSF) in the proportion of 1 g plant material per 13 cm^3 of buffer. Homogenate was filtered through *Miracloth* and then centrifuged for 15 min at 30 000 *g*. Activity of the studied enzymes and concentrations of GABA, L-alanine and L-glutamate were analyzed in supernatant.

Determination of glutamate decarboxylase activity: Decarboxylation was carried out at 30 °C in the mixture containing in a volume of 0.4 cm^3 3 mM L-glutamate, 20 μM PLP, 50 mM K-phosphate buffer, pH 5.8, and the enzyme. The enzyme was preincubated in the mixture without L-glutamate for 10 min. The reaction was initiated by addition of amino acid and stopped after 60 min by adding 0.1 cm^3 of 0.5 M HCl. The mixtures were centrifuged (10 min at 12 500 *g*), supernatant filtered through 0.45 μm filter, diluted with 0.1 M HCl and mixed with the OPA reagent (0.2 % *o*-phthalaldehyde, 20 % methanol, 0.13 M 2-mercaptoethanol in 0.4 M borate buffer, pH 12.5) in the proportion of 1:1.2. After 2 min, 0.02 cm^3 of the solution were loaded on the HPLC column *Waters Nova-Pak* C_{18} (3.9/150 mm). Separation was performed according to the modified method Waters Auto-Tag™ OPA. γ-Aminobutyrate derivative was detected and determined with *WATERS 474* scanning fluorescence detector (*Waters*, Milford, MA, USA).

Determination of the GABA aminotransferase activity: Transamination was carried out at 30 °C in the mixture containing in a volume of 0.4 cm^3 50 mM Tris-HCl buffer (pH 8.4) 20 mM GABA, 10 mM 2-oxoglutarate or 20 mM pyruvate, 3 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM PLP. The enzyme was preincubated in the mixture without 2-oxoacid for 10 min. The reaction was started by addition of 2-oxoacid and stopped after 60 min by adding 0.1 cm^3 of 0.5 M HCl. Further treatment was similar to that in determining decarboxylase activity. The OPA derivatives of the amino acid products were separated, detected and determined as above.

Determination of GABA, L-alanine, L-glutamate, and protein content: Proteins from prepared homogenates (see above) were removed using 6 mM sulfosalicylic acid. After centrifugation (10 min at 12 500 *g*) supernatant was neutralized with 1 M NaOH, filtered (0.45 μm filter), mixed with the OPA reagent in the proportion of 1:1.2 and GABA, L-alanine and L-glutamate derivatives were separated on HPLC column, detected and determined as above. The protein content was determined according to Bradford (1976) using bovine serum albumin as a standard.

Results and discussion

Within first 4 h of the experiment, WSD of plants treated with 20 % PEG 6000 markedly increased in comparison with the control (Fig. 1*A*). After 100 h, WSD of the stressed plants was 27 % while that of the control plants was 10 %.

Twenty eight hours after application of 20 % PEG 6000 GABA concentration increased over twofold comparing with the control (Fig. 1*B*). Changes in the

GABA content in leaves of plants under osmotic shock caused by PEG 6000 were not studied yet. GABA was the only amino acid, from the free amino acids pool that increased significantly in tomato seedlings leaves grown under stress caused by 140 mM NaCl (Bolarín *et al.* 1995). This increase was not observed in leaves of the wild tomato species which is more tolerant to salinity (Bolarín *et al.* 1995).

No significant changes in L-glutamate and L-alanine concentration were found in stressed wheat plants comparing with the control (data not presented). Both amino acids participate in the γ-aminobutyrate pathway.

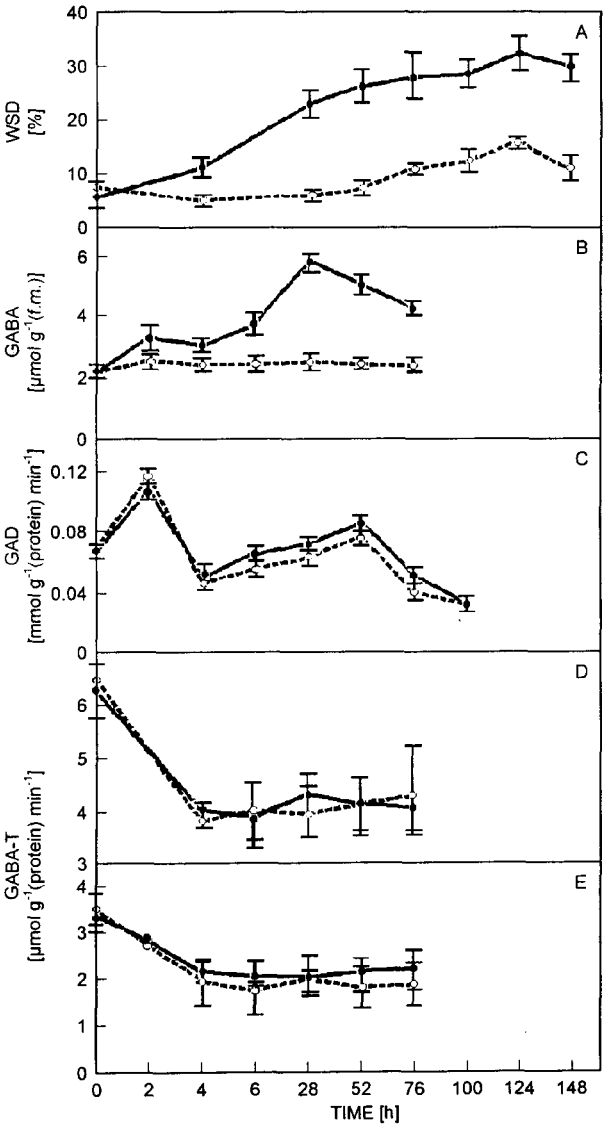

Fig. 1. Time course of WSD (*A*), GABA concentrations (*B*), GAD activity (*C*), GABA-T activity with 2-oxoglutarate (*D*) and GABA-T activity with pyruvate (*E*) in wheat seedlings grown under control medium (*dashed line*) and 20 % PEG 6000 (*full line*). Values are means of 3 replicates. *Vertical bars* represent ± SE of the mean (not shown when smaller than the symbol).

The first is a GAD substrate and a substrate and product of GABA-T while the second is a substrate and a product of GABA-T (Shelp *et al.* 1999). This observation additionally points to a specific role of GABA in metabolism associated with osmotic stress.

The expression of the gene encoding GAD the enzyme catalysing GABA synthesis is probably regulated at the transcription and translation levels (Shelp *et al.* 1999). Literature data show that GAD, an enzyme sensitive to calmoduline stimulation (Akama *et al.* 2001, Rhodes *et al.* 1999) is also activated by H^+ (Bown and Shelp 1997) and L-glutamate (Scott-Taggart *et al.* 1999). Therefore, one might expect that the observed increase of GABA concentration should be accompanied by the enhanced GAD activity in stressed plants. Such effect was not found. Two hours after the beginning of the experiment, decarboxylase activity increased almost twice in both types of samples. Later on, enzyme activity decreased and distinct differences between stressed and control plants were not observed (Fig. 1*C*).

Differences were also not found for two activities (with pyruvate or 2-oxoglutarate as an amino group acceptor) of GABA-T, the second of the studied enzymes of the GABA pathway. After 4 h of stress both aminotransferase activities decreased almost two times in both stressed and not stressed seedlings (Fig. 1*D,E*). Later on the two activities remained at a constant level (Fig. 1*D,E*).

It seems that the increased GABA concentration was a result of the inhibition of GABA degradation in the γ-aminobutyrate shunt. The third, not studied enzyme of the pathway – succinic semialdehyde dehydrogenase is inhibited by ATP, ADP, AMP and NADH (Bush and Fromm 1999). Under various stress conditions, concentrations of these compounds change in different way. For example, with the lack of oxygen, $NADH/NAD^+$ and ADP/ATP ratios increase (Bush and Fromm 1999). This has an inhibiting effect on dehydrogenase activity and results in accumulation of its substrate, *i.e.*, succinic semialdehyde. Van Cauvenberghe and Shelp (1999) found that succinic semialdehyde at a concentration of 2 mM markedly inhibited GABA-T. Inhibition of this aminotransferase by the semialdehyde might thus directly contribute to the increase of γ-aminobutyrate concentration in wheat leaves cells. Additional factor enhancing this effect could be the very high ratio of specific activity of GAD to that of GABA-T which was 11:1 when 2-oxoglutarate served as amino group acceptor (Fig. 1*C,D*) and 19:1 when pyruvate played this role (Fig. 1*C,E*). Observed variations in activities of GAD and GABA-T (Fig. 1*C,D,E*) may suggest that medium change can influence the control mechanisms of two investigated enzymes.

Different pH sensitivity of two GABA-T activities (GABA: 2-oxoglutarate and GABA: pyruvate) and unequal ratio of these activities in various tissues of the

Table 1. Inhibition [%] of GABA: 2-oxoglutarate and GABA: pyruvate aminotransferase activities by various compounds. The wheat seedlings homogenates were preincubated with inhibitor and amino acid substrate or only with amino acid in the assay buffer. The reaction was started by addition of 2-oxoacid. Means of three separate experiments.

Inhibitor	Concentration [mM]	GABA: 2-oxo-glutarate	GABA: pyruvate
Control	0	0	0
Aminooxyacetate	0.01	0	92
	0.10	59	92
	1.00	81	93
3-chloro-L-alanine	0.10	42	65
	1.00	72	85
p-hydroxymercuri-benzoate	0.01	7	36
	0.10	20	62
	1.00	45	81

same plant (Shelp *et al.* 1995) allows to suspect that GABA-T, as many other aminotransferases, exists as isoforms localized in different subcellular fractions (Ireland and Lea 1999). Van Cauvenberghe and Shelp (1999) separated these two GABA-T isoforms on ion-exchange column. It is the first and so far the only direct experimental evidence that different enzymatic proteins are responsible for two GABA aminotransferase activities.

In this paper we attempted to clarify the question of occurrence of various GABA-T forms by inhibitory studies. Applied compounds differed in their way of action on enzyme. Aminooxyacetate and 3-chloro-L-alanine block carbonyl group of pyridoxal phosphate - coenzyme of aminotransferases (Morino and Tanase 1985); *p*-hydroxymercuribenzoate (PHMB) reacts with reactive -SH groups of enzymatic protein (Paszkowski 1995). Observed inhibition of transamination by aminooxyacetate and 3-chloro-L-alanine (Table 1) points to the cooperation of GABA-T with PLP. Inhibition of γ-aminobutyrate aminotransferase(s) by PHMB (Table 1) proves that essential hydrosulfide groups do exist in the enzyme molecule. Degree of inhibition obtained for activities with two different oxoacid substrates and the same inhibitor distinctly differed in each of three cases (Table 1). Therefore, the results of inhibitory studies provide the evidence for the occurrence of two different proteins catalysing GABA transamination in wheat leaves, each displaying different specificity towards the analysed amino group acceptors: pyruvate or 2-oxo-glutarate. This would confirm the suggestion of Van Cauvenberghe and Shelp (1999) that there are at least two pathways for GABA metabolism in plants.

References

Akama, K., Akihiro, T., Kitagawa, M., Takaiwa, F.: Rice (*Oryza sativa*) contains a novel isoform of glutamate decarboxylase that lacks an authentic calmodulin-binding domain at the C-terminus. - Biochim. biophys. Acta **1522**: 143-150, 2001.

Bolarín, M.C., Santa Cruz, A., Cayuela, E., Peréz-Alfocea, F.: Short-term solute changes in leaves and roots of cultivated and wild tomato seedlings under salinity. - J. Plant Physiol. **147**: 463-468, 1995.

Bown, A.W., Shelp, B.J.: The metabolism and functions of γ-aminobutyric acid. - Plant Physiol. **115**: 1-5, 1997.

Bradford, M.M.: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Breitkreuz, K.E., Shelp, B.J., Fisher, W.N., Shwacke, R., Rentsch, D.: Identification and characterization of GABA, proline and quaternary ammonium compound transporters from *Arabidopsis thaliana*. - FEBS Lett. **450**: 280-294, 1999.

Bush, K., Fromm, H.: Plant succinic semialdehyde dehydrogenase. Cloning, purification, localization in mitochondria, and regulation by adenine nucleotides. - Plant Physiol. **121**: 589-597, 1999.

Chen, Y., Baum, G., Fromm, H.: The 58-kD calmodulin binding glutamate decarboxylase is a ubiquitous protein in *Petunia* organs and its expression is developmentally regulated. - Plant Physiol. **106**: 1381-1387, 1994.

Chung, L., Bown, A.W., Shelp, B.J.: The production and efflux of 4-aminobutyrate in isolated mesophyll cells. - Plant Physiol. **99**: 659-664, 1992.

Fisher, W.N., Andre, B., Rentsch, D., Krolkiewicz, S., Tegeder, M., Breitkreutz, K., Fromer, W.B.: Amino acid transport in plants. - Trends Plants Sci. **5**: 188-195, 1988.

Hampe, C., Lundgren P., Daniels, T., Hammerle, L., Marcovina, S., Lernmark, A.: A novel monoclonal antibody specific for the N-terminal end of GAD 65. - J. Neuroimmunol. **113**: 63-71, 2001.

Ireland, R.J., Lea, P.: The enzymes of glutamine, glutamate, asparagine and aspartate metabolism. - In: Singh, B.K. (ed.): Plant Amino Acids. Pp. 49-109. Marcel Dekker, New York - Basel - Hong Kong 1999.

Kathiresan, A., Tung, P., Chinnappa, C.C., Reid, D.M.: γ-Aminobutyric acid stimulates ethylene biosynthesis in sunflower. - Plant Physiol. **115**: 129-135, 1997.

Miazek, A., Bogdan, J., Zagdańska, B.: Effect of water deficit during germination of wheat seeds. - Biol. Plant. **44**: 397-403, 2001.

Morino, Y., Tanase, S.: Quasisubstrates and irreversible inhibitors of aspartate aminotransferase. - In: Christen, P., Metzler, D.E. (ed.): Transaminases. Pp. 251-265. John Wiley and Sons, New York 1985.

Paszkowski, A.: The hydrosulfide groups of glutamate: glyoxylate and serine: glyoxylate aminotransferases from rye (*Secale cereale* L.) seedlings. - Acta Physiol. Plant. **17**: 85-90, 1995.

Rai, V.K.: Role of amino acids in plant responses to stresses. - Biol. Plant. **45**: 481-487, 2002.

Ramputh, A.L., Bown, A.W.: Rapid gamma-aminobutyric acid synthesis and the inhibition of growth and development of oblique-banded leaf-roller larvae. - Plant Physiol. **111**: 1349-1352, 1996.

Rhodes, D., Versules, P.E., Sharp, R.E.: Role of amino acids in abiotic stress resistance. - In: Singh, B.K. (ed.): Plant Amino Acids. Pp. 319-356. Marcel Dekker, New York - Basel - Hong Kong 1999.

Schwacke, R., Grallath, S., Breitkreutz, K.E., Stransky, E., Stransky, H., Fromer, W.B., Rentsch, D.: LeProT1, a transporter for proline, glycine, betaine and γ-aminobutyric acid in tomato pollen. - Plant Cell **11**: 377-391, 1999.

Scott-Taggart, C.P., Van Cauwenberghe, O.R., McLean, M.D., Shelp, B.J.: Regulation of γ-aminobutyric acid synthesis *in situ* by glutamate availability. - Physiol. Plant. **106**: 363-369, 1999.

Shelp, B.J., Bown, A.W., McLean, M.D.: Metabolism and functions of gamma-aminobutyric acid. - Trends Plant Sci. **4**: 446-452, 1999.

Shelp, B.J., Walton, C.S., Snedden, W.A., Tuin, L.G., Oresnik, I.J., Layzell, D.D.: GABA shunt in developing soybean seeds is associated with hypoxia. - Physiol. Plant. **94**: 219-228, 1995.

Solomon, P.S., Oliver R.P.: The nitrogen of tomato leaves apoplast increases during infections by *Cladosporium fulvum*. - Planta **213**: 241-249, 2001.

Van Cauwenberghe, O.R., Shelp, B.J.: Biochemical characterization of partially purified GABA: pyruvate transaminase from *Nicotiana tabacum*. - Phytochemistry **52**: 575-581, 1999.