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Characterization of the γ-aminobutyric acid shunt pathway and oxidative damage in *Arabidopsis thaliana pop 2* mutants under various abiotic stresses

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Abstract

In the present study, three *Arabidopsis thaliana pop2* mutant lines with different T-DNA insertions in a gene coding γ-aminobutyric acid transaminase (GABA-TA) were screened for seed germination percentage, stress-induced oxidative damage, and GABA content and metabolism under various abiotic stresses including high temperature (42 ºC), low temperature (4 ºC), salinity (NaCl), and osmotic stress (mannitol). All mutant lines showed a decreased germination under all the stress treatments with a significant reduction in the *pop*2-*1* and *pop2-3* mutant lines. Content of GABA and MDA increased significantly in all *pop2* mutants and wild type (WT) seedlings in response to all the treatments. However, content of GABA and MDA was lower in all *pop2* mutants comparing to the WT under the same treatments. GABA increased already after 30 min and increased significantly after 2 h at 42 °C especially in the *pop2-3* and WT seedlings. In response to the cold treatment, GABA content increased up to 4-fold compared to the control in all *pop2* mutants and WT seedlings. In response to the NaCl treatment, GABA accumulated slightly in the WT and all *pop2* mutants. On the contrary, GABA content increased significantly in the *pop2, pop2-1,* and *pop2-3* mutants and WT under all mannitol treatments.

Additional key words: abiotic stress, cold, GABA, heat, osmotic stress, salinity.

Introduction

Stress tolerance in plants involves a variety of genetic and molecular adaptive mechanisms (Bohnert *et al.* 1995) such as osmoregulation (McNeil *et al.* 1999), calcium signaling (Hetherington and Brownlee 2004), and accumulation of γ-aminobutyric acid (GABA) (Steward 1949, Mayer *et al.* 1990, Breitkreuz and Shelp 1995, Locy *et al.* 2000). The metabolic pathway that converts glutamate to succinate through GABA is called the GABA shunt. The GABA shunt pathway in plants contains a cytosolic enzyme glutamate decarboxylase (GAD) and mitochondrial enzymes GABA transaminase (GABA-TA) and succinate semialdehyde dehydrogenase (SSADH). Each of these enzymes appears to have a regulatory role in GABA metabolism beside its formal catalytic role in the GABA shunt pathway (Shelp *et al.* 1999, Bouché and Fromm 2004). A rapid accumulation of GABA in response to heat, cold, ultra-violet (UV) radiation, drought, salinity, anoxia, mechanical stress, and wounding has been demonstrated (Steward 1949, Mayer *et al.* 1990, Breitkreuz and Shelp, 1995, Bown and Shelp, 1997, Shelp *et al.* 1999, Locy *et al.* 2000, Petrivalsky *et al.* 2007, Al-Quraan 2015). Beside its direct role as stress metabolite (Kinnersley and Turano 2000, Bouche and Fromm 2003, Bouché *et al.* 2003a,b, Palanivelu *et al.* 2003, Shelp *et al.* 2006), it has been suggested that GABA regulates gene expression including the expression of the 14-3-3 gene family (Lancien and Roberts 2006).

A high content of GABA is crucial for pollen tube growth, which emphasizes the role of this non-protein amino acid in intercellular signaling (Palanivelu *et al.* 2003). In *Arabidopsis thaliana*, a gene *pollen-pestil-*

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Abbreviations: GABA - γ-aminobutyric acid; GABA-TA - γ-aminobutyric acid transaminase; GAD - glutamate decarboxylase; MDA - malondialdehyde; MS - Murashige and Skoog; $NADP^+$ - nicotinamide adenine dinucleotide phosphate; *pop2- pollen-pestil-incombatability2*; ROS - reactive oxygen species; SSADH - succinate semialdehyde dehydrogenase; TCA - tricarboxylic acid cycle; WT - wild type.

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incombatability2 (*POP2*) encodes GABA-TA that degrades GABA (Van Cauwenberghe *et al.* 2002). The *pop2* mutant is unable to produce a functional GABA-TA enzyme (Yu *et al.* 2006). The homozygous *pop*2 mutant is self-sterile even though the pollen of this mutant can germinate and penetrate the stigma. The pollen tube guidance was found to be abnormal (Ma 2003) and increases the content of GABA occurring along the pollen tube path in the wild type, whereas the *pop*2 mutant shows no such a normal GABA gradient. This disturbance leads to growth retardation and misguidance of *pop2* pollen tubes in *pop*2 pistils (Bouché *et al.* 2003a,b, Ma 2003, Palanivelu *et al.* 2003). The *pop*2 phenotype demonstrates the role of GABA in plant reproduction and development (Bouché *et al.* 2005).

In the present study, we explored the response of the *Arabidopsis thaliana* wild type and *pop2* mutants under various abiotic stresses including high and low temperatures, salinity, and osmotic stress, and the role of GABA-TA in these abiotic stresses responses was examined with respect to seed germination, GABA accumulation, and oxidative damage.

Materials and methods

Plants and growth conditions: Accession numbers for three alleles of AT3G22200 locus were the *pop2* mutant (line 1, SALK_007661), *pop2-1* mutant (line 2, CS6385) and *pop2-3* mutant (line 3, CS6387). All seeds were obtained from the Arabidopsis Biological Research Stock Center, the Ohio State University, Columbus, USA. The seeds were surface disinfected with 6 % (m/v) sodium hypochlorite for 10 min followed by five washes with sterile distilled water. The seeds were plated in Petri dishes on a sterile Murashige and Skoog (1962; MS) medium (pH 5.7) supplemented with 2 % (m/v) sucrose and solidified with 1.2 % (m/v) agar. The seedlings were grown in a growth chamber under a continuous irradiance of 40 μmol m^{-2} s^{-1} (provided by cool white fluorescent lamps) and a temperature of 25 ºC using a synthetic soil (*Promix*, *Premier Tech Horticulture*, Rivière-du-Loup, Canada).

RT-PCR analysis for *GABA-TA* in *pop2* mutants: The absence of corresponding transcripts of the GABA-TA gene in the three alleles of the *pop2* mutants used in this study was confirmed by RT-PCR analysis (Fig. 1). Total

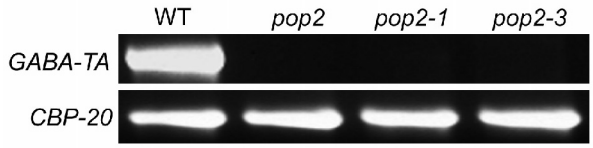

Fig. 1. Molecular characterization of *pop2* mutants and the wild type (WT). A RT-PCR analysis with *GABA-TA* specific primers showing the absence of *GABA-TA* transcripts in all *pop2* mutants (n = 3; *CBP-20* - internal control).

RNA was extracted from shoots of 10-d-old plants using an *IQeasy™ Plus* RNA extraction mini kit (*Intron Biotechnology*, Gyeonggi-do, Korea) according to the manufacturer's instructions. One-step reverse transcriptase-PCR (RT-PCR) was performed using primer pairs specific for the GABA-TA gene (a forward primer 5′-GCATGTCTGGGAACTCCACT-3′ and a reverse primer 5′-AGGCAAACGCTTTAACTCCA-3′; Miyashita and Good 2008) and *CBP*20 as internal control. A *SuperScript*™ *III One-step* RT-PCR system (*Intron Biotechnology*) with platinum® *Taq* DNA polymerase was used according to the manufacturer's instructions. RT-PCR amplification products were separated on 2 % (m/v) agarose gels and stained with ethidium bromide.

Seed germination and thermotolerance assay: Seed sensitivity to cold and heat treatments was performed according to Hong and Vierling (2000) with the following modifications. For cold treatment, surface sterilized seeds of WT and *pop2* mutants were suspended in 0.5 cm^3 of 4 ºC sterile distilled water and incubated at 4 ºC for 24 h. For heat treatment, surface sterilized seeds of the WT and *pop2* mutants were suspended in 0.5 cm^3 of sterile distilled water and incubated at 42 ºC in a closed water bath block for 2 and 3 h. Each tube contained 50 seeds. At the end of the incubation, the seeds were plated on Petri dishes and allowed to grow as described above for 7 d. Emergence of radicles from germinating seeds was recorded and compared to the respective control.

Seed sensitivity to salinity and osmotic stress: A hundred and fifty surface sterilized seeds of the WT and each of the *pop2* mutants were plated on an MS medium supplemented with 0, 75, 100, and 150 mM NaCl or 0, 100, 200, and 300 mM mannitol and allowed to grow under the conditions described above for 10 d.

Oxidative damage: One set of two-week old seedlings of the WT and *pop2* mutants (50 seeds each) were exposed to 4 ºC for 6 and 12 h separately in a growth chamber. Another set was heat-treated at 42 ºC for 2 h. The content of malondialdehyde (MDA) of the seedlings was determined using the thiobarbiturate reactive substances assay (Heath and Packer 1968). Seven-day-old seedlings grown on an MS medium under continuous irradiance were transferred onto MS plates supplemented with 0, 70, 100, and 150 mM NaCl or 0, 100, 200, and 300 mM mannitol. The plates were incubated for next 7 d. Content of MDA was determined. Plant tissues after each treatment were frozen in liquid N_2. Tissue (0.50 g) was ground in a 1.5 cm^3 microfuge tube and 0.5 cm^3 of 0.5 % (m/v) thiobarbituric acid in 20 % (m/v) trichloroacetic

acid, and 0.5 cm^3 of 175 mM NaCl in 50 mM Tris-HCl at pH 8 were added to the ground tissue. Tubes were then heated to 90 ºC for 25 min. The samples were centrifuged at 38 000 *g* for 20 min and the supernatant was collected. The absorbance of the supernatant was measured at 532 nm using a *752S UV-VIS* spectrophotometer, *Biocoteck*, Ningbo, China. Content of MDA was determined from a standard curve.

GABA extraction and determination: For cold treatments, two-week-old seedlings of the WT and *pop2* mutants grown on MS agar plates at 25 ºC were transferred to a growth chamber set at 4 ºC for 1, 3, 6, and 12 h, separately. For heat treatments, the WT and *pop2* seedlings were exposed to 42 ºC for 30 min, 1, and 2 h. Seven-day-old WT and *pop2* mutant seedlings were transferred to MS media supplemented with 0, 70, 100 and 150 mM NaCl or 0, 100, 200 and 300 mM mannitol and grown for next 7 d. GABA was extracted from frozen tissues of the WT and *pop2* mutants according to Zhang and Bown (1997) with the following modifications. Harvested roots and shoots were ground separately in 1.5 cm^3 microfuge tubes in liquid N_2 until a fine powder was obtained. Methanol (0.4 cm^3) was added to each tube and the samples were mixed for 10 min. Liquid from the samples was removed by vacuum drying, and 0.5 cm^3 of 70 mM lanthanum chloride was added to each tube. The tubes were shaked for 15 min and subsequently centrifuged at 38 000 *g* for 5 min. The supernatants were transferred to new tubes, mixed with 0.16 cm^3 of 1 M KOH, shaken for 10 min, and centrifuged at 38 000 *g* for 5 min. Content of GABA was determined according to Zhang and Bown (1997) in a reaction mixture containing 0.05 cm^3 of a sample extract, 0.014 cm^3 of 4 mM $NADP^+$, 0.019 cm^3 of 0.5 M potassium pyrophosphate (pH 8.6), 0.01 cm^3 of α-ketoglutarate, and 0.01 cm^3 of *GABASE* (GABA-aminotransferase plus succinic semi-aldehyde dehydrogenase, *Sigma-Aldrich* Taufkirchen, Germany). *GABASE* (2 U mm^{-3}) was suspended in 0.1 M potassium pyrophosphate, pH 7.2, containing 12.5 % (v/v) glycerol, and 5 mM β-mercaptoethanol). A change in absorbance at 340 nm after addition of α-ketoglutarate was recorded after 90 min incubation at 25 ºC using a microplate reader (*BioTek* power wave, *Max200R*, Vermont, USA). The content of GABA was determined using an NADPH standard curve.

Statistical analysis: Three plates with 50 seeds each were usually used for each treatment. Each data point was expressed as mean ± standard deviation (SD) of three independent experiments. The values were compared and analyzed by two-way analysis of variance (*ANOVA*) using least significant difference (LSD) multiple comparison tests on the means. Where differences were reported, they were at a 95 % confidence level ($P < 0.05$). Regression analyses between germination percentage and GABA content and between germination percentage and MDA content were performed and are shown in Table 1 Suppl. All analyses were performed using the statistical software package *SPSS version 15.*

Results and discussion

The seeds of the *pop2* (line 1), *pop2-1* (line 2) and *pop2-3* (line 3) mutants exposed to 4 °C for 24 h showed a 77, 65, and 40 % successful germination, respectively, compared to a 91 % germination in the WT seeds (Table 1). The *pop2* exposed to 42 ºC showed a 13 % reduction in germination after 2 h and a 30 % reduction after 3 h. The germination of the *pop2-1* and *pop2-3* seeds was significantly ($P < 0.05$) reduced by 30 and 75 % after the 42 ºC treatment for 2 h and by 70 and 85 % after 3 h, respectively, (Table 1). The seed germination oversensitivity of the *pop2-1* and *pop2-3* mutants to the temperature stress supports a hypothesis that GABA-TA may be involved in defensive pathways that contribute to successful seed germination and plant survival in response to low and high temperature treatments. In fact, the impairment of the GABA shunt pathway results in oversensitivity of *Arabidopsis* to various abiotic stresses such as low and high temperatures (Locy *et al.* 2000, Ludewig *et al.* 2008, Al-Quraan *et al.* 2011, 2012). Prell *et al*. (2002) reported that GABA as a sole source of carbon and nitrogen stimulates growth in *Rhizobium leguminosarum*, which in turn highlights the role of GABA shunt in plant growth and metabolism. The oversensitivity of the *pop2-1* and *pop2-3* mutants to the heat and cold treatments indicates that a GABA-TA defect impaired GABA accumulation that might be required for growth and implicates that GABA is involved in intracellular signaling especially during seed germination under temperature stress.

The germination of *pop2, pop2-1*, and *pop2-3* was more inhibited as result of salinity comparing to the WT

Table 1. Germination [%] of seeds under cold, heat, NaCl, and mannitol treatments in *pop2* mutants and wild type (WT) of *Arabidopsis thaliana*. Treated seeds were platted on a solid MS medium and allowed to germinate at 25 °C under continuous irradiance for 7 d (* - significant at $P < 0.05$, $n = 9$).

Stresses	Treatments	*pop2*	*pop2-1*	*pop2-3*	WT
	control	94	89	91	98
4 ºC	24 h	77	67*	41*	91
42 ºC	2 h	87	67*	26*	93
	3 h	72	31*	16*	88
NaCl	75 mM	72	56*	36*	91
	100 mM	38	37*	13*	87
	150 mM	14	10*	7*	84
Mannitol	100 mM	86	65*	60*	95
	200 mM	79	69*	50*	90
	300 mM	59	55*	17*	85

(Table 1). The germination reductions of *pop2*, *pop2-1*, and *pop2-3* seeds reached 30, 40, and 60 % with 75 mM NaCl, 60, 63, and 85 % with 100 mM NaCl, and 85, 90, and 95 % with 150 mM NaCl, respectively. On the other hand, the WT seeds germinated normally (85 - 95 %) after the same treatments. A similar pattern of germination tolerance/sensitivity was observed in response to the mannitol treatments (Table 1). The seed germination of the *pop2-1* and *pop2-3* seeds were reduced significantly ($P < 0.05$) in response to the osmotic stress. After 10 d of treatment, the *pop2-1* and *pop2-3* seeds germinated up to 75 and 70 % at 100 mM mannitol, 70 and 50 % at 200 mM mannitol, and 50 and 20 % at 300 mM mannitol, respectively. However, the *pop2* mutants showed only a 40 % reduction under 300 mM mannitol, and the WT a 15 % reduction under the same treatment (Table 1). This data indicate that the *pop2* mutant had a high sensitivity to the salt and osmotic stresses, which implicates that a functional GABA-TA enzyme and GABA shunt integrity play a vital role in metabolic stability and salt and osmotic stress tolerance in *Arabidopsis*.

In plants, different functions have been suggested to the GABA shunt and GABA metabolism such as osmoregulation and glutamate homeostasis control (Shelp *et al.* 1999, Masclaux-Daubresse *et al.* 2002, Renault *et al.* 2010, Al-Quraan *et al.* 2013). The oversensitivity of the *pop2-1* and *pop2-3* mutants to salt and osmotic stresses may be connected to a signaling role of GABA. It has been shown that GABA is involved in regulation of nitrogen metabolism (Beuve *et al.* 2004) and *14-3-3* genes expression (Lancien and Roberts 2006). Renault *et al.* (2010) found that GABA-TA is important in *Arabidopsis* root nitrogen and carbon metabolism under NaCl treatments.

Table 2. Content of MDA [nmol mg^{-1}(f.m.)] under cold, heat, NaCl, and mannitol treatments in *pop2* mutants and WT of *A. thaliana* seedlings (* - significant at $P < 0.05$, $n = 9$).

Stresses	Treatments	*pop2*	*pop2-1*	*pop2-3*	WT
	control	0.011	0.013	0.018	0.146
4 ºC	6 h	0.014	0.018	0.019	0.190
	12 h	0.300*	0.600*	0.900*	0.230*
42 ºC	2 h	0.110*	0.220*	0.180*	0.300*
NaCl	75 mM	0.026	0.028	0.038	0.157
	100 mM	0.038	0.035	0.056	0.182
	150 mM	0.045	0.068*	0.098*	0.226*
Mannitol	100 mM	0.032	0.036	0.065	0.150
	200 mM	0.039	0.049	0.082	0.167
	300 mM	0.339*	0.452*	0.871*	0.183

The content of MDA increased significantly in all the *pop2* mutants and the WT seedlings in response to all the treatments (Table 2). However, the content of MDA was lower in all the *pop2* mutants compared to the WT under the same treatments except for the cold treatment. This supports a hypothesis that GABA shunt enzymes, specifically GABA-TA, have a role in mediating stress tolerance broadly and is a component of this limiting oxidative stress damage in all stresses investigated (Steward 1949, Mayer *et al.* 1990, Breitkreuz and Shelp 1995, Bown and Shelp 1997, Shelp *et al.* 1999, Locy *et al.* 2000, Petrivalsky *et al.* 2007, Al-Quraan 2015). The data are consistent with a role for GABA-TA in oxidative stress damage. However, the lower content of MDA in the *pop2* mutants compared to the WT seedlings supports a hypothesis that additional aspects of the GABA shunt, such as SSADH or other anaplerotic pathway(s), pleiotropically mediate the oxidative stress response of the GABA-TA mutants.

In response to the heat treatment, the *pop2* and *pop2-3* mutants showed a 6- to 10-fold increase of MDA ($P < 0.05$) compared to a 2- to 17-fold increase of MDA ($P < 0.05$) in the WT and *pop2-1,* respectively, under the same treatment. In response to the cold treatment, MDA content increased ($P < 0.05$) 46-fold in the *pop2-1*, 50-fold in *pop2-3,* 27-fold in *pop2*, and 1.5-fold in WT seedlings at 4 ºC after 12 h (Table 2). The production of the ROS by the *pop2-1* and *pop2-3* mutants during the heat and cold treatments was consistent with the seed germination of the same mutants under the same treatments.

In response to the NaCl treatment, MDA accumulated in the *pop2* mutants was higher compared to the WT under the same NaCl concentration (Table 1). The accumulation of MDA in the *pop2*, *pop2-1*, and *pop2-3* mutants increased up to 2-fold at 75 mM NaCl, 3-fold at 100 mM NaCl, and 4- to 5-fold at 150 mM NaCl, whereas in the WT seedlings, the MDA accumulation increased 1.5-fold under the same treatments. By comparison, all the *pop2* mutants accumulated more MDA under all the mannitol treatments ($P < 0.05$; Table 2). In response to 100 mM mannitol, 3- to 5-fold increase in MDA content was observed in all the *pop2* mutants compared to a non-significant change in the WT seedlings. At 200 mM mannitol, all the *pop2* mutant seedlings accumulated MDA up to 4- to 5-fold higher ($P < 0.05$) compared to no increase in the WT seedlings. However, all the mutants showed significant increases of MDA content ($P < 0.05$) under 300 mM mannitol, *pop2* and *pop2-1* a 31- to-35-fold increase and *pop2-3* a 49-fold increase compared to a 1.3-fold increase in the WT (Table 2).

The oxidative damage phenotype and the production of the ROS in *pop2*, *pop2-1*, and *pop2-3* as result of the NaCl and mannitol treatments were in agreement with the germination sensitivity of the same mutants under the same treatments. The MDA accumulation in the *pop2* mutants and WT observed in this study might be related to disruption of carbon and nitrogen mitochondrial metabolic pathways and changes in membrane integrity and stability as result of the stress treatments. These results implicate ROS production as part of a reduced temperature tolerance and salt/osmotic oversensitivity of the *pop2* mutants especially in the *pop2-1* and *pop2-3* mutants. Reactive oxygen species production has been

observed after various stresses in many plants, and thus our findings are consistent with those previously reported (*e.g.*, Foyer *et al.* 1994, Harndahl *et al.* 1998, Lu and Zhang 1999). Bouché *et al*. (2003a) showed that functional SSADH is vital to suppress H_2O_2 accumulation under abiotic stresses, which in turn compromises the function of the GABA shunt that leads to ROS accumulation and cell death under high irradiance and heat stresses. Since the GABA shunt supplies a metabolic intermediate to the tricarboxylic acid cycle, an impaired shunt and respiration under abiotic stress may enhance the accumulation of ROS and oxidative damage.

Table 3. Content of GABA [nmol mg^{-1}(f.m.)] under cold, heat, NaCl, and mannitol treatments in *pop2* mutants and WT of *A. thaliana* seedlings (* - significant at $P < 0.05$, $n = 9$).

Stresses	Treatments	*pop2*	*pop2-1*	*pop2-3*	WT
	control	0.07	0.06	0.11	0.67
4 ºC	1 h	0.06	0.11	0.06	0.25
	3 h	0.08	0.12	0.15*	0.35
	6 h	0.12*	0.15*	0.18*	0.42*
	12 h	0.16*	0.19*	0.25*	0.50*
42 ºC	30 min	0.07	0.06	0.11	0.67*
	1 h	0.09	0.09	0.13	1.92*
	2 h	0.11*	0.19*	0.50*	2.33*
NaCl	75 mM	0.07	0.06	0.09	0.15
	100 mM	0.08	0.09	0.10	0.24*
	150 mM	0.09*	0.13*	0.13*	0.31*
Mannitol	100 mM	0.09	0.13	0.09	0.69*
	200 mM	0.11	0.18*	0.21*	0.98*
	300 mM	0.26*	0.31*	0.86*	1.43*

In general, GABA accumulation in all the *pop2* mutants was lower than in the WT seedlings under all the stress treatments. Furthermore, GABA increased already after 30 min, and after 1 h at 42 °C, it increased 2.4-fold in all the *pop2* mutants compared to 16-fold in the WT. After 2 h at 42 °C, GABA content increased 9.3-fold in the *pop2-3* mutant and 19-fold in the WT. In response to the cold treatments, GABA content increased 0.5- to 4-fold compared to the control in all the *Arabidopsis pop2* mutants and WT seedlings (Table 3). The maximum increase occurred at 4 ºC for 12 h in *pop2-3* (5.4-fold) and the WT (4-fold). Generally, GABA increased up to 2.7-fold in all the *pop2* mutants and WT compared to the control in response to the NaCl treatments (Table 3). Furthermore, the *pop2-1* and *pop2-3* mutants showed a higher GABA content compared to the *pop2* mutant under 75, 100, and 150 mM NaCl. On the contrary, *pop2, pop2-1, pop2-3*, and WT accumulated more GABA under all the mannitol treatments. Gamma-aminobutyric acid increased 1.6- to 5-fold in *pop2*, 3- to 7-fold in *pop2-1*, 2- to 16-fold ($P < 0.05$) in *pop2-3*, and 6- to 12-fold in WT seedlings under 100, 200, and 300 mM mannitol treatments.

The lower GABA accumulation in the *pop2* mutants compared to the WT under all the stresses is consistent with the stress impairment of the GABA shunt pathway. As result of an impaired GABA-TA function in the GABA shunt, the *pop2* seedlings likely redirected glutamate metabolism to maintain cellular homeostasis of C:N ratio. Theoretically, glutamate can participate in the TCA cycle through the GABA shunt (Michaeli *et al.* 2011) or by cytosolic transamination of glutamate and movement of 2-oxyglutarate to mitochondrial matrix (Fait *et al.* 2008), or by mitochondrial uptake of glutamate through glutamate transporters and conversion to 2-oxyglutarate inside the matrix (Forde and Lea 2007). Since glutamate is used in various transamination reactions producing 2-oxyglutarate, it is involved directly in regulation of C:N splitting and amino acid metabolism. Minocha *et al*. (2014) reported that plants respond to stress by enhancing N assimilation through production and accumulation of polyamines from glutamate to cause overall cellular homeostasis of C and N. In addition, an increase in these polyamines can lead to a decrease of glutamate catabolism in the direction of GABA production.

Mazzucotelli *et al*. (2006) found that during cold acclimation and freezing in wheat and barley, GABA shunt metabolite accumulation and shunt enzyme gene expressions were triggered in response to a low temperature, whereas GABA buildup and GAD action were linked to the severity of the stress. Al-Quraan *et al*. (2012) reported that accumulation of GABA shunt metabolites as result of cold and heat treatments in some *Arabidopsis* calmodulin mutants might be associated with activation of GAD after exposure to temperature stress in response to a low cytoplasmic pH and intracellular damage. Since all the *pop2* mutants and WT in this study showed a similar pattern of GABA accumulation under the cold and heat treatments, the GABA shunt may have a protective role in response to extreme temperature stresses.

Gamma-aminobutyric acid shunt pathway has been suggested to be involved in osmoregulation, ion homeostasis, and signaling under salt and osmotic stresses (Shelp *et al.* 1999, Boucheand Fromm 2004, Xing *et al.* 2007, Renault *et al.* 2010). The content of GABA varied between the *pop2* genotypes under the salt and osmotic treatments (Table 3). The high GABA accumulation under salt stress might be involved in adaptation to the salt and osmotic stresses and signaling in response to the salinity, and it might serve as osmoprotectant in response to the high osmolarity and low water availability under the mannitol treatments. Beside its role in osmoregulation, GABA may be involved in ion balance and homeostasis. Al-Quraan *et al.* (2011) reported that a significant increase of GABA, alanine, and glutamate content occur in roots and shoots of *cam5-4* and *cam6-1* calmodulin mutants in response to paraquat (0.5, 1, and 3 μM). However, they increase only in the root tissue of the *cam5-4* and *cam6-1* mutants in response to H_2O_2 (200, 500 μM, and 1 mM). In agreement with this study, Renault *et al*. (2010) reported that GABA metabolism is activated by NaCl treatment

since all genes of GABA metabolism (*GAD* and *GABA-T*) are up-regulated under salt treatments, which in turn reflects the importance of GABA accumulation under salt stress. In addition, Al-Quraan *et al.* (2013) reported that salt and osmotic stresses in wheat seedlings cause a high accumulation of GABA metabolites. Bartyzel *et al*. (2003) showed that a significant increase in GABA occurrs in wheat seedlings after exposure to 20 % polyethylene glycol 6000 for 28 h. Akçay *et al*. (2012) found that a cytoplasmic male sterility mutant and the WT of *Nicotiana sylvestris* plants show a better growth under a long term salinity due to GABA accumulation and metabolism. Gamma-aminobutyric acid shunt metabolites may have a crucial role in osmoregulation, linking N and C metabolisms and signaling in response to salinity and osmotic stress. The accumulation of GABA especially in the *pop2-3* mutant suggests the prominent metabolic function of GABA-TA in C/N metabolism and GABA signaling in the *Arabidopsis* tissues under the NaCl and mannitol treatments. The sensitivity of the *pop2* mutants to all the stress treatments was negatively correlated (r = -0.780 to -0.999) with GABA accumulation and also negatively correlated (r = -0.851 to -0.999) with the MDA accumulation (Table 1 Suppl.), which led to a decreased ability of the plants to germinate under the stress.

The low GABA accumulation in the *pop2* mutants under the stress compared to the wild type led to an increased accumulation of MDA, which in turn indicates that GABA might be involved in oxidative damage protection through maintaining redox equilibrium and preventing accumulation of the ROS and cell death in response to abiotic stresses. Gamma-aminobutyric acid might be indirectly involved in scavenging hydroxyl radicals and superoxide ions produced during cellular metabolism, regulating redox buffer changes, and signaling inside the cell under all the stresses used in this study.

In conclusion, this study provides an insight into the involvement of the GABA shunt pathway under the abiotic stresses resulting from the function of GABA-TA. In addition, GABA transamination *via* GABA-TA is an enzymatic step that can potentially regulate GABA metabolism, osmoregulation, and adaptation under extreme temperatures and ionic and osmotic stresses in *Arabidopsis* seedlings.

References

Akçay, N., Bor, M., Karabudak, T., Özdemir, F., Türkan, I.: Contribution of gamma amino butyric acid (GABA) to salt stress responses of *Nicotiana sylvestris* CMSII mutant and wild type plants. - J. Plant. Physiol. **169**: 452-458, 2012.

Al-Quraan, N.A.: GABA shunt deficiencies and accumulation of reactive oxygen species under UV treatments: insight from *Arabidopsis thaliana* calmodulin mutants. - Acta. Physiol. Plant. **37**: 1-11, 2015.

Al-Quraan, N.A., Locy, R.D., Singh, N.K.: Implications of paraquat and hydrogen peroxide-induced oxidative stress treatments on the GABA shunt pathway in *Arabidopsis thaliana* calmodulin mutants. - Plant. Biotechnol. Rep. **5**: 225-234, 2011.

Al-Quraan, N.A., Locy, R.D., Singh, N.K.: Heat and cold stresses phenotypes of *Arabidopsis thaliana* calmodulin mutants: regulation of gamma-aminobutyric acid shunt pathway under temperature stress. - Int. J. Plant. Biol. **3**: 9-17, 2012.

Al-Quraan, N.A., Sartawe, F.A., Qaryouti, M.M.: Characterization of γ-aminobutyric acid metabolism and oxidative damage in wheat (*Triticum aestivum* L.) seedlings under salt and osmotic stress. - J. Plant. Physiol. **170**: 1003-1009, 2013.

Bartyzel, I., Pekzar, K., Paszkowski, A.: Functioning of the gamma-aminobutyrate pathway in wheat seedlings affected by osmotic stress. - Biol. Plant. **47**: 221-225, 2003.

Beuve, N., Rispail, N., Laine, P., Cliquet, J-B., Ourry, A., Le Deunff, E.: Putative role of γ-aminobutyric acid (GABA) as a long-distance signal in up-regulation of nitrate uptake in *Brassica napus* L. - Plant Cell Environ. **27**: 1035-1046, 2004.

Bohnert, H.J., Nelson, D.E., Jensen, R.G.: Adaptation to environmental stresses. - Plant Cell **7**: 1099-1111, 1995.

Bouché, N., Fait, A., Bouché, D., Moller, S.G., Fromm, H.: Mitochondrial succinate-semialdehyde dehydrogenase of the gamma-aminobutyrate shunt in required to restricts levels of reactive oxygen intermediates in plants. - Proc. nat. Acad. Sci. USA **100**: 6843-6848, 2003a.

Bouché, N., Fromm, H.: GABA in plants: just a metabolite? - Trends Plant. Sci. **9**: 110-115, 2004.

Bouché, N., Lacombe, B., Fromm, H.: GABA signaling: a conserved and ubiquitous mechanism. - Trends Cell Biol. **13**: 607-610, 2003b.

Bouché, N., Yellin, A., Snedden, W.A., Fromm, H.: Plant-specific calmodulin-binding proteins. - Annu. Rev. Plant. Biol. **56**: 435-466, 2005.

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

Breitkreuz, K.E., Shelp, B.J.: Subcellular compartmentalization of the 4-aminobutyrate shunt in protoplasts from developing soybean cotyledons. - Plant. Physiol. **108**: 99-103, 1995.

Fait, A., Fromm, H., Walter, D., Galili, G., Fernie, A.R.: Highway or byway: the metabolic role of the GABA shunt in plants. - Trends Plant. Sci. **13**: 14-19, 2008.

Forde, B.G., Lea, P.J.: Glutamate in plants: metabolism, regulation, and signaling. - J. exp. Bot. **58**: 2339-2358, 2007.

Foyer, C.H., Descourvieres, P., Kunert, K.J.: Protection against oxygen radicals: an important defense mechanisms studies in transgenic plants. - Plant. Cell Environ. **17**: 507-523, 1994.

Harndahl, U., Hall, R., Osteryoung, K.: The chloroplastic small heat shock protein undergoes oxidation-dependent conformational changes and may protect plants from oxidative stress. - Cell Stress Chaperon. **4**: 129-138, 1998.

Heath, R.L., Packer, L.: Photoperoxidation in isolated chloroplasts. - Arch. Biochem. Biophys. **125**: 189-198, 1968.

Hetherington, A., Brownlee, C.: The generation of Ca^{2+} signal in plants. - Annu. Rev. Plant. Biol. **55**: 401-428, 2004.

Hong, S.W., Vierling, E.: Mutants of *Arabidopsis thaliana* defective in the acquisition of tolerance to high temperature

stress. - Proc. nat. Acad. Sci. USA **97**: 4392-4397, 2000.
Kinnersley, A.M., Turano, F.J.: Gamma aminobutyric acid (GABA) and plant responses to stress. - Crit. Rev. Plant. Sci. **19**: 479-509, 2000.
Lancien, M., Roberts, M.R.: Regulation of *Arabidopsis thaliana* 14-3-3 gene expression by gamma-aminobutyric acid. - Plant. Cell Environ. **29**: 1430-1436, 2006.
Locy, R.D., Wu, S-J., Bisnette, J., Barger, T.W., McNabb, D., Zik, M., Fromm, H, Singh, N.K., Cherry, J.H. (ed.): The Regulation of GABA Accumulation by Heat Stress in *Arabidopsis*. (NATO Science Series. Vol. 83). - Springer, Dordrecht, 2000.
Lu, C., Zhang, J.: Effects of water stress on PS II photochemistry and its thermostability in wheat plants. - J. exp. Bot. **50**: 1199-1206, 1999.
Ludewig, F., Hüser, A., Fromm, H., Beauclair, L., Bouché, N.: Mutants of GABA transaminase (*POP2*) suppress the severe phenotype of succinic semialdehyde dehydrogenase (*ssadh*) mutants in *Arabidopsis*. - PLoS. ONE **3**: 1-10, 2008.
Ma, H.: Plant reproduction: GABA gradient, guidance and growth. - Curr. Biol. **13**: R834-R836, 2003.
Masclaux-Daubresse, C., Valadier, M.H., Carrayol, E., Reisdorf-Cren, M., Hirel, B.: Diurnal changes in the expression of glutamate dehydrogenase and nitrate reductase are involved in the C/N balance of tobacco source leaves. - Plant Cell Environ. **25**: 1451-1462, 2002.
Mayer, R.R., Cherry, J.H., Rhodes, D.: Effects of heat shock on amino acid metabolism of cowpea cells. - Phytochemistry **94**: 796-810, 1990.
Mazzucotelli, E., Tartari, A., Cattivelli, L., Forlani, G.: Metabolism of γ-aminobutyric acid during cold acclimation and freezing and its relationship to frost tolerance in barley and wheat. - J exp. Bot. **57**: 3755-3766, 2006.
McNeil, S., Nuccio, M., Hanson, A.: Betaines and related osmoprotectants targets for metabolic engineering of stress resistance. - Plant. Physiol. **120**: 945-949, 1999.
Michaeli, S., Fait, A., Lagora K., Nunes-Nesi, A., Grillich, N., Yellin, A., Bar, D., Khan, M., Fernie, A.R., Turano, F.J., Fromm, H.: A mitochondrial GABA permease connects the GABA shunt and the TCA cycle, and is essential for normal carbon metabolism. - Plant. J. **67**: 485-498, 2011.
Minocha, R., Majumdar, R., Minocha, S.C.: Polyamines and abiotic stresses in plants: a complex relationship. - Front. Plant Sci. **5**: 175, 2014.
Miyashita, Y., Good, A.G.: Contribution of the GABA shunt to hypoxia-induced alanine accumulation in roots of *Arabidopsis thaliana*. - Plant Cell Physiol. **49**: 92-102, 2008.
Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 472-497, 1962.
Palanivelu, R., Brass, L., Edlund, A.F., Preuss, D.: Pollen tube growth and guidance is regulated by POP_2, an *Arabidopsis* gene that controls GABA levels. - Cell **114**: 47-59, 2003.
Petrivalsky, M., Brauner, F., Luhova, L., Gagneul, D., Sebela, M.: Aminoaldehyde dehydrogenase activity during wound healing of mechanically injured pea seedlings. - J. Plant Physiol. **164**: 1410-1418, 2007.
Prell, J., Boesten, B., Poole, P., Priefer, U.B.: The *Rhizobium leguminosarum bv. viciae* VF39 gamma-aminobutyrate (GABA) aminotransferase gene (*gabT*) is induced by GABA and highly expressed in bacteroids. - Microbiology **148**: 615-623, 2002.
Renault, H., Roussel, V., El-Amrani, A., Arzel, M,, Renault, D., Bouchéreau, A., Deleu, C.: The *Arabidopsis pop2-1* mutant reveals the involvement of GABA transaminase in salt stress tolerance. - BMC Plant. Biol. **10**: 20, 2010.
Shelp, B.J., Bown, A., Faure, D.: Extracellular γ-aminobutyrate mediated communication between plants and other organisms. - Plant. Physiol. **142**: 1350-1352, 2006.
Shelp, B.J., Bown, A.W., McLean, M.D.: Metabolism and functions of gamma-aminobutyric acid. - Trends Plant Sci. **4**: 446-452, 1999.
Steward, G.R.: γ-Aminobutyric acid: a constituent of the potato tuber? - Science **110**: 439-440, 1949.
Van Cauwenberghe, O.R., Makhmoudov, A., McLean, M.D., Clark, S.M., Shelp, B.J.: Plant pyruvate-dependent gamma-aminobutyrate transaminase: identification of an *Arabidopsis* cDNA and its expression in *Escherichia coli*. - Can. J. Bot. **80**: 933-941, 2002.
Xing, S.G., Jun, Y.B., Hau, Z.W., Liang, L.Y.: Higher accumulation of gamma-aminobutyric acid induced by salt stress though stimulating the activity of diamine oxidases in *Glycine max* (L) Merr. roots. - Plant. Physiol. Biochem. **45**: 560-566, 2007.
Yu, G., Liang, J., He, Z., Sun, M.: Quantum dot-mediated detection of γ-aminobutyric acid binding sites on the surface of living pollen protoplasts in tobacco. - Chem. Biol. **13**: 723-731, 2006.
Zhang, G., Bown, A.W.: The rapid determination of gamma-aminobutyric acid. - Phytochemistry **44**: 1007-1009, 1997.