Redox regulation of free amino acid levels in *Arabidopsis*

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Abiotic stresses induce oxidative stress, which modifies the level of several metabolites including amino acids. The redox control of free amino acid profile was monitored in wild type and ascorbate or glutathione deficient mutant *Arabidopsis* plants before and after hydroponic treatment with various redox agents. Both mutations and treatments modified the size and redox state of the ascorbate (AsA) and/or glutathione (GSH) pools. The total free amino acid content was increased by AsA, GSH and H_2O_2 in all 3 genotypes and a very large (3-fold) increase was observed in the GSH-deficient pad2-1 mutant after GSH treatment compared to the untreated wild type plants. Addition of GSH reduced the ratio of amino acids belonging to the glutamate family on a large scale and increased the relative amount of non-proteinogenic amino acids. The latter change was due to the large increase in the content of alpha-aminoadipate, an inhibitor of Glu transport. Most of the treatments increased the Pro content, which effect was due to the activation of genes involved in Pro synthesis. Although all studied redox compounds influenced the amount of free amino acids and a mostly positive, very close ($r>0.9$) correlation exists between these parameters, a special regulatory role of GSH could be presumed due to its more powerful effect. This may originate from the thiol/disulphide conversion or (de)glutathionylation of enzymes participating in the amino acid metabolism.

Abbreviations – Aaa, α-aminoadipic acid; AsA, ascorbic acid; Cysta, cystathione; DHA, dehydroascorbate; DTT, dithiotreitol; GSH, glutathione; GSSG, glutathione disulphide; NPPAs, non-proteogenic amino acids; OAT, ornithine aminotransferase; PDH, proline dehydrogenase; P5CR, pyrroline-5-carboxylate reductase; P5CDH, delta-1-pyrroline-5-carboxylate dehydrogenase; P5CS1, delta

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1-pyrroline-5-carboxylate synthase 1; P5CS2, delta 1-pyrroline-5-carboxylate synthase 2.

Introduction

Redox status has a major role in the regulation of metabolism and is controlled by metabolic processes at the same time (Geigenberger and Fernie 2014). The proper redox status during growth and development under optimal and adverse environmental conditions is maintained by the antioxidant system through the removal of the excess of reactive oxygen species (ROS; Dietz 2008, Suzuki et al. 2012, Kocsy et al. 2013, Considine and Foyer 2014). Accumulation of ROS, such as superoxide radical, hydrogen peroxide, hydroxyl radical and singlet oxygen in high concentration may cause serious injuries or even plant lethality under stress conditions (Bartosz 1997). However, a moderate increase in ROS content may have positive functions as well since, as signaling molecules, they activate the defense mechanisms in plants. Two main antioxidants are glutathione and ascorbate; they control the level of H_2O_2 as the components of the ascorbate-glutathione cycle (Foyer and Noctor 2011). In addition, both compounds are involved in the regulation of growth and development (Noctor et al. 2012). These functions were confirmed in ascorbate- and glutathione-deficient Arabidopsis mutants (vtc2-1, pad2-1) (Müller-Moulé et al. 2004, Dubreuil-Maurizi et al. 2011).

The relationship between the metabolism of the tripeptide GSH (γ-Glu-Cys-Gly) and amino acids was shown in transgenic poplar where overexpression of the gene encoding chloroplastic γ-Glu-Cys synthetase increased not only the GSH level, but also the concentration of several amino acids (Noctor 1998). In addition, induction of proline synthesis by transformation with the *pyrroline-5-carboxylate reductase* gene (P5CR) in soybean was accompanied by reduced GSH levels (Kocsy et al. 2005). This observation can be explained by the greater use of the common precursor, glutamate, for proline synthesis, and it indicates the coordinated control of antioxidant and amino acid levels. The redox control of amino acid levels was also shown in catalase-deficient *Arabidopsis* mutants in which the increase in H_2O_2 level was accompanied by greater concentration of several amino acids (Noctor et al. 2015).

Besides their use for protein synthesis, several proteinogenic amino acids play important role in various physiological processes. Serine has a crucial role in plant development and metabolism (Ros et al. 2014) and in responding to biotic and abiotic stress effects (Ho and Saito 2001, Benstein et al. 2013). This proteinogenic amino acid has catalytic function in enzymes, participates in biosynthesis of several important biomolecules such as other amino acids, phospholipids and sphingolipids. Glutamate is another fundamental amino acid that has a central role in amino acid metabolism of plants because many other amino acids, such as proline, arginine, ornithine, γ-aminobutyric acid are formed from glutamate. These reactions are catalyzed by multispecific aminotransferases (Forde and Lea 2007). A signaling function for glutamate in plants was proposed; therefore, it can affect many physiological processes including the

growth and development of plants (Forde and Lea 2007). Its involvement in stress response is indicated by its increased level after water stress in rice (Yang et al. 2000), dehydration in *Sporobolus stapfianus* (Martinelli et al. 2007) and high salt concentration in maize (Boldizsár et al. 2013). Proline has a dominant role in the control of plant development, flowering, abiotic and biotic stress response and signaling (Trovato et al. 2008, Hayat et al. 2012). Numerous reports have shown that proline content was increased by different stress factors such as drought, high salinity, high light, heavy metals, osmotic and oxidative stress (Verbruggen and Hermans 2008). Proline is able to scavenge ROS and maintain the redox balance in plant cells since proline biosynthesis from glutamate can serve as an alternative source of $NADP^+$ when the rate of Calvin cycle is reduced under stress situation (Szabados and Savouré 2010). In addition to the proteinogenic amino acids, there are several non-proteinogenic amino acids (NPPAs) that play important roles in protecting against stress, signaling and nitrogen storage in plants (Vranova et al. 2010). NPPAs also have antiherbivory, antimicrobial and allelochemical activity like toxins too (Bell 2003).

Although some of the cited data indicated the redox control of free amino acid levels, this regulation was not tested in a complex system earlier. In the present study the proposed relationship between changes in the ascorbate- and glutathione-dependent redox status and free amino acid levels was investigated by the modification of antioxidant levels using the components of AsA-GSH cycle and a synthetic reductant, dithiothreitol (DTT) in wild type *Arabidopsis* plants and mutants having reduced AsA and GSH levels.

Materials and methods

Plant material and growth conditions

Wild type *Arabidopsis* (Col-0) plants and glutathione-deficient (*pad2-1*) (Parisy et al. 2007) and ascorbate-deficient (*vtc2-1*) (Conklin et al. 2000) mutants were studied. The seedlings were grown in a soil block (Jiffy, Jiffy Products S.L. Ltd.) in a photoperiod of 8 h, at 120 μmol m^{-2} s^{-1}, 23/22°C and 70/75% RH in a growth chamber. The 5-week old seedlings were transferred onto half-strength modified Hoagland-solution. After a 5-day long adaptation phase, various reductants in 4 mM concentration (GSH, AsA, DTT) and an oxidant (hydrogen-peroxide in 10 mM concentration) were added to the nutrient solution. Based on our previous experiments in maize and wheat, these compounds are suitable for the modification of the GSH-dependent redox environment (Kellős et al. 2008, Gulyás et al. 2014). Their concentrations used in the present experiments were determined in preliminary experiments in which a concentration range (1–10 mM) with different treatment durations (1–7 d) was used. Previously, DTT was used successfully as a very strong reductant in several redox experiments (Shaikhali et al. 2008, Gulyás et al. 2014). After a 3-day treatment, samples were collected for biochemical analysis and gene expression studies. There were 3 independent experiments with 3 parallel samples each. The induced redox changes were monitored by measuring the amount of reduced and oxidized forms of glutathione and ascorbate by

HPLC and the detection of hydrogen peroxide levels by spectrophotometer.

Determination of ascorbate

Shoot samples of 500 mg fresh weight were ground with liquid nitrogen in a mortar and extracted with 3 ml of 5% meta-phosphoric acid. In the supernatant, AsA content and total ascorbate concentration were determined by HPLC using an Alliance 2690 system (Waters, Milford, MA, USA) equipped with a photodiode array (PDA) detector (W996, Waters, Milford, MA, USA). For the latter analysis, dehydroascorbate (DHA) was reduced with dithiotreitol (DTT) to ascorbic acid. The concentration of DHA was estimated from the difference between the amount of total ascorbic acid and AsA (Soltész et al. 2011).

Analysis of glutathione

The plant material was ground with liquid nitrogen in a mortar after which 1 ml of 0.1 M HCl was added to 200 mg plant sample. Total glutathione content was determined after a reduction with dithiothreitol and derivatization with monobromobimane (Kocsy et al. 2000). For the detection of glutathione disulphide (GSSG), the reduced glutathione (GSH) was blocked with *N*-ethylmaleimide and the excess of *N*-ethylmaleimide was removed with toluene (Kranner and Grill 1996). GSSG was reduced and derivatized as described for total thiols. The samples were analyzed by reverse-phase HPLC (Waters, Milford, MA, USA) using a W474 scanning fluorescence detector (Waters). The amount of GSH was calculated as the difference between the amounts of total glutathione and GSSG.

Measurement of hydrogen peroxide content

Hydrogen peroxide content was determined by FOX1 method as described previously (Kellos et al. 2008).

Determination of protein and free amino acid content

The total protein content was estimated according to the method of Bradford (Bradford 1976) using bovine serum albumin as standard as described earlier (Soltész et al. 2011).

For the measurement of amino acids, shoot samples of 500 mg fresh weight were crushed in liquid nitrogen and extracted with 2 ml cold 10% trichloroacetic acid for 1 h with gentle agitation on a shaker (C. Gerhardt GmbH & Co. KG, Germany) at room temperature. Each sample was filtered through a 0.2 µm pore membrane filter (Sartorius AG, Germany). The biochemical analysis was carried out on an automatic amino acid analyzer (Ingos Ltd., Czech Republic) equipped with an Ionex Ostion LCP5020 cation-exchange column (22 cm × 0.37 cm). The free amino acids were separated by stepwise gradient elution using a Li^+-citric buffer system (Ingos Ltd., CzechRepublic). Colorimetric detection was accomplished at 570 nm and 440 nm (for Pro) after post-column derivatization with ninhydrin reagent.

Gene expression studies

Total RNA was extracted with Direct-zol™ RNA Miniprep Kit (Zymo Research) according to the manufacturer's instructions. Reverse transcription was performed using M-MLV Reverse Transcriptase and Oligo (dT) 15 primer (Promega) as described by the manufacturer. The gene expression levels were determined with real-time qRT-PCR using a CFX96 thermocycler (Bio-Rad) with custom-made primers (Appendix S1.). The samples originated from 3 independent experiments with 3 repetitions each. The relative quantities of the individual transcripts were calculated with the ΔΔCt method using the *Actin2* (Wang et al. 2014) housekeeping gene (TAIR ID: AT3G18780.2) for normalization (Livak and Schmittgen 2001). As the mutations did not affect the expression level of the investigated genes except for *P5CS2* (*delta 1-pyrroline-5-carboxylate synthase 2*) and *P5CR* in *vtc2-1,* so all the other data were given as values relative to their own control in every genotype.

Statistical analysis

Data from three independent experiments were evaluated and standard deviations are indicated on the figures. Statistical analysis was performed by using one-way analysis of variance (SPSS program). Least significant difference method was used for *post hoc* analysis. The relationships between the various parameters were checked by correlation analysis (Excel program).

Results

Changes in the amount and redox state of ascorbate and glutathione

Compared to the wild-type plants, (Col-0) the AsA-content in the AsA-deficient *vtc2-1* mutant was lower by 75% under control conditions as expected (Fig. 1a). Interestingly, it was restored to control levels detected in Col-0 when treated by GSH, and the other treatment also increased its amount but up to smaller degree. In addition, the GSH treatment also increased the AsA levels in the other two genotypes and H_2O_2 in *pad2-1* compared to Col-0 control. Although GSH also increased DHA levels in Col-0 and *vtc2-1*, this change was not significant (Fig. 1b). The AsA/DHA ratio was lower in *vtc2-1* after all of the treatments than in Col-0 control (Fig. 1c). This ratio was also reduced by GSH in Col-0. In Col-0 all the treatments, except for H_2O_2, decreased GSH concentration compared to the untreated control (Fig. 1d). In the GSH-deficient *pad2-1* mutant, the GSH content was lower by 82% than in Col-0 under control conditions. This difference only slightly changed after the AsA, DTT and H_2O_2 treatments. However, treatment with GSH restored the GSH content to the levels detected in Col-0 control. GSSG content was increased in every genotype by GSH and in Col-0 by AsA compared to the Col-0 control (Fig. 1e). The GSH/GSSG ratio was lower in *pad2-1* and greater in *vtc2-1* under control conditions compared to the Col-0 (Fig.1f). It was decreased significantly by GSH and AsA in all 3 genotypes and by DTT and H_2O_2 in

Col-0 compared to the Col-0 control.

Changes in hydrogen peroxide content

After all treatments, except for DTT, the amount of H_2O_2 was greater in *pad2-1* than in Col-0 control (Appendix S2). In the other two genotypes there was no significant difference compared to the Col-0 control.

Effect of redox treatments on total soluble protein concentration

The amount of total soluble protein was greater in *pad2-1* and *vtc2-1* plants than in Col-0 under control conditions (Appendix S3). Its level was increased by all treatments except for DTT in Col-0 plants and for GSH and H_2O_2 in *vtc2-1* mutant compared to the untreated Col-0 plants. The protein content was decreased by H_2O_2 in *vtc2-1* and by GSH in *pad2-1* mutant compared to the Col-0 control plants.

Effect of the redox compounds on free amino acid levels

In our experiments, concentrations of 28 free amino acids were measured. Without treatments, mutant plants have higher total free amino acid levels than the Col-0 ones (Fig. 2a).The majority of the treatments induced a significant increase in the total free amino acid level in every genotype except for AsA in Col-0 plants and for DTT in all three genotypes compared to the untreated Col-0 plants. Their amount was 2 to 4-fold greater in GSH-treated *pad2-1* mutants than in all of the other samples. Besides the total amount of free amino acids, the ratio of the various amino acid families was also affected by the treatments (Fig. 2b). The ratio of glutamate family was about 50% and the ratio of aspartate family was about 30% – both with and without treatments in the examined three genotypes except for GSH application. After GSH treatment, the ratio of these families decreased, and the ratio of other amino acids consisting mainly of non-proteinogenic amino acids exhibited a huge increase (from a few percent to 40%).The ratio of the serine family increased after the redox treatments except for GSH in all genotypes. The ratio of pyruvate family was lower after GSH and H_2O_2 treatments in the mutants compared to the Col-0 plants.

Regarding the individual amino acids, the concentration of the most investigated amino acids was increased significantly by GSH treatment in *pad2-1* mutant compared to Col-0 control plants (Fig.3, Appendix S4). Based on the hierarchical clustering related to the treatments, GSH induced similar alterations in the other two genotypes but at smaller scale. The other two investigated component of the AsA-GSH cycle, AsA and H_2O_2, also had great effect on the amino acid pattern and they formed a mixed hierarchical group. Interestingly, the synthetic reductant, DTT did not affect the amount of most amino acid in the three studied genotypes except for cysteine compared to the untreated Col-0. The mutations or treatments alone did not influence the amount of most amino acids except for H_2O_2. Regarding the grouping of amino acids, the level of most of them in the glutamate family (Glu, Gln, Pro, Orn) exhibited

similar concentration changes after the various treatments as shown by the clustering. In the other groups with great concentration changes, members of all 5 major amino acid families (Val, Arg, Lys, Leu, Phe, Ser, His, Ile, 3mHis) were present. Among the NPPAs, the Aaa (α-aminoadipic acid), the Cysta (cysthationine) and the Aba (α-aminobutyric acid) were increased dramatically by GSH treatment. The other amino acids exhibited only smaller changes and only after fewer treatments. The changes in the total free amino acid content were similar to those represented by the members of the glutamate family, which had a 50% ratio compared to the other families. The major amino acids in this family were Glu, Gln, Pro and GABA. The ratio of proline was increased in all genotypes after most treatments while the ratio of the Gln or Glu decreased compared to the untreated Col-0 (Fig.4.). AsA, GSH and H_2O_2 induced an increase in the ratio of arginine. In serine family Ser was the major amino acid (90%) in all samples except for DTT-treated ones in which a large increase in Cys content (from 10–15% to 25–50%) was observed (Fig. 5). In aspartate family Asp, Asn and Thr together reached 90%, and their ratios were only slightly or not affected by the treatments (Appendix S5a). In pyruvate family, the ratio of Ala reached 70–75% under control conditions, and its ratio decreased by a paralleled increase in Val and Leu after most treatments (Appendix S5b). Two aromatic amino acids, Tyr and Phe, were present in detectable amounts. Phe content reached 65–75% in Col-0 and *vtc2-1* and 35% in *pad2-1* under control conditions (Appendix S6a). The ratio of Phe was increased by the treatments in *pad2-1*. In the other two genotypes, DTT decreased the ratio of Phe. In addition, 8 other amino acids (His and 7 non-proteinogenic amino acids) could be detected. Among them, the ratio of alpha-aminoadipic acid increased from 15–20% to 85–90% by GSH in all 3 genotypes (Appendix S6b). A 3- and a 5-fold increase in the ratio of this amino acid was observed after AsA and H_2O_2 treatment in *vtc2-1* mutant, respectively.

Correlation analysis revealed a relationship between the amount or redox state of the ascorbate and glutathione and the concentration of most free amino acids in *pad2-1* mutant, but not in the Col-0 or *vtc2-1* plants (Appendix S7a–c). This correlation was positive (except for the GSH/GSSG and Asn comparison) and very close ($r>0.9$) for total free amino acid, Orn, Glu, Ile, Asn and 3 mHis content (at least for 3 redox parameters from the studied 7). Regarding the individual redox parameters, the level of the following ones showed very close positive correlation with the concentration of amino acids (their number is given in parentheses) in the investigated genotypes: Col-0 – AsA (2); GSSG (2); *pad2-1* – AsA (9); GSH (13); GSSG (8); *vtc2-1*- AsA (2); DHA (3) (Appendix S7d). Interestingly, when the correlation analysis was prepared based on the treatments, very close correlations were observed between the concentration of the studied redox parameters (at least for 3) and amino acids in untreated control (Thr, Asp, Leu, Ala, 3mHis, Baiba, Aba), AsA-treated (Pro, Glu, Gln, Met, Thr, Asn, Ala, Phe, Baiba, Aba, Cit), DTT- treated (Pro, Orn, Glu, Gln, Cys, Ser, Lys, Thr, Asp, Leu, Tyr, His, Baiba, Cysta, Aba, Aaa) and H_2O_2-treated (Arg, Cys, Met, Lys, Thr, Asn, Tyr, Phe, Baiba, Bala, Aaa) plants, but not in the case of the GSH-treatment

(Appendix S7e–i). Regarding the individual redox parameters, the following ones showed a very close positive (+) or negative (–) correlation with the concentration of amino acids (their number is given in parentheses after the direction of correlation) with the concentration of amino acids after the individual treatments: Control – DHA (+: 1; –: 8); GSH (+ : 6; –: 5); H_2O_2 (+: 5 ; –: 3); GSH treatment – AsA/DHA (+: 19); H_2O_2 (+: 17); AsA treatment – DHA (+: 7; –: 2); GSH (+: 2; –: 6); GSSG (+: 2; –: 8); GSH/GSSG (+: 12); H_2O_2 (+: 13; –: 1); DTT treatment – DHA (+: 7; –: 3); GSH (+: 5; –: 3); GSSG (+: 4; –: 5); GSH/GSSG (+ 6; – 6); H_2O_2 (+ 4; – 5); H_2O_2 treatment – AsA (+ 2; – 8); DHA (+ 6; – 2); AsA/DHA (– 9); H_2O_2 (+ 2; – 7) (Appendix S7j).

Regarding the relationship between the content of various amino acids, a very close correlation was found between the amounts of several amino acids belonging to the aspartate family in all 3 genotypes (Appendix S7a–c). When the comparison was based on the treatments, a very close, mostly positive (only 3.5% of the 841 correlations were negative) correlation was found between the concentration of nearly all amino acids after GSH treatment, except for Met, Val, Baiba, Bala and Aba (Appendix S7e–h).

Redox regulation of proline metabolism related genes

As proline was very sensitive to the redox treatments and it was described as a susceptible compound for stress effects, it was verified whether the observed changes in Pro levels derives from the alterations in the expression of the related metabolic genes. Pro has two biosynthesis pathways. *OAT* (*ornithine aminotransferase*) is the main gene in one of these pathways encoding an enzyme, ornithine-delta aminotransferase, which produces proline from ornithine via pyrroline-5-carboxylate. The expression level of *OAT* was increased by GSH treatment in all genotypes. DTT and AsA treatment caused an increase in *vtc2-1* plants (Fig. 6). The *P5CR* responded with a slight increase for AsA treatment in Col-0 and *vtc2-1* plants and for H_2O_2 in every genotype. In the other biosynthesis pathway, the *P5CS1 (delta 1-pyrroline-5-carboxylate synthase 1)* transcript level was increased by AsA treatment in Col-0, by H_2O_2 in *pad2-1* and by GSH treatment in *vtc2-1* mutant. Interestingly the *P5CS2* expression was much greater without treatments and after GSH addition in *vtc2-1* mutant compared to the other two genotypes. H_2O_2 treatment increased its transcription in *pad2-1* mutants. In ascorbate-deficient plants, the expression level of *P5CS2* decreased significantly after H_2O_2 treatment compared to the untreated control plants. Genes encoding P5CDH (delta-1-pyrroline-5-carboxylate dehydrogenase) and PDH (proline dehydrogenase) enzymes taking part in proline catabolism were also studied. The expression of *P5CDH* was greater after H_2O_2 treatment in all of the genotypes following AsA treatments in both mutants and after addition of GSH in *pad2-1* mutant. *PDH* transcription was increased by GSH and AsA in Col-0, and by GSH and H_2O_2 in *pad2-1*.

The correlation analysis based on the genotypes revealed a very close positive correlation ($r>0.9$)

between several investigated redox parameters (at least for 3 from 7) and the amount of Orn and Glu and the expression of the gene encoding OAT in *pad2-1* mutant, but not in the other two genotypes (Appendix S7a–c). The analysis based on the treatments demonstrated a very close correlation (partly negative, partly positive with no clear tendency) between the redox compounds and the expression of *P5CS1*, *P5CS2*, *P5CDH* and *P5CR* in untreated control and GSH-treated plants, the amount of Pro, Orn, Glu and expression of *P5CS*', *P5CS2*, *P5CDH*, *PDH* and *OAT* genes in AsA-treated plant, the concentration of Pro and transcript levels of *P5CDH* and *OAT* genes in DTT-treated plants and the expression of *PDH* gene after H_2O_2 treatment (Appendix S7e–i).

Discussion

Changes in the size and redox state of the ascorbate and glutathione pools

In order to see the possible regulatory effect of the AsA-GSH cycle on the concentrations and ratios of free amino acid levels, two approaches were used. According to the first one, the AsA-deficient *vtc2-1* and the GSH-deficient *pad2-1 Arabidopsis* mutants were compared with the wild type Col-0 plants under control conditions, without any pharmacological treatments. Consistent with the literature data, much lower AsA and GSH levels (less than 20% of the value detected in Col-0) were detected in *vtc2-1* and *pad2-1* mutants, respectively (Conklin et al. 2000, Parisy et al. 2007).

According to the second approach, both of the Col-0 and mutants plants were treated with AsA, GSH and H_2O_2 (which are the components of the AsA-GSH cycle), and with a strong reductant, DTT, which is not synthesized in plants. While the amount and redox state of AsA was not affected by most of the treatments, the concentration and redox state of GSH was modified by most of them. This difference can be explained by the smaller size of glutathione pool making it more sensitive to the pharmacological treatments. It should be noted that the amount of GSSG was greatly increased by GSH in all 3 genotypes. The excess of GSH could inhibit the enzymes reducing the oxidized forms of these thiols as described for glutathione reductase in rat liver (Chung et al. 1991). The coordinated control of the AsA and GSH concentrations is indicated by the fact that GSH could increase AsA level in the *vtc2-1* mutant, and AsA could elevate GSH content in *pad2-1* mutant. In addition, there was a very close correlation between AsA and GSH contents if the analysis was based on the genotypes. Taken together, the present experimental system proved to be suitable to modify the amount and redox state of AsA and GSH and examine the effect of these changes on the free amino acid levels.

Effect of ascorbate- and glutathione-dependent redox alterations on amino acid pattern

Since the total protein content was not or only slightly (maximum 30% changes) influenced by the mutations or the applied treatments, the observed greater changes in the total free amino acid contents do

not derive from the altered protein metabolism. The total free amino acid content was affected by mutations and treatments with the components of the AsA-GSH cycle. However, DTT did not affect it, which indicates a special control of amino acid levels by the AsA-GSH cycle and not a simple redox regulation. This special effect could be explained by GSH-related thiol/disulphide conversion or (de)glutathionylation of amino acids in enzymes involved in amino acid metabolism or by AsA-dependent coenzymes (Kocsy et al. 2013).

A coordinated redox-dependent regulation of AsA, GSH and amino acid contents was shown earlier during ozone-induced oxidative stress in poplar (Dumont et al. 2014). In this experiment ozone increased the level of 7 free amino acids while it decreased the concentration of 13 amino acids. In contrast to ozone, the greater H_2O_2 content in catalase-deficient *Arabidopsis* mutants resulted in higher levels of 21 free amino acids form the 23 investigated ones (Noctor et al. 2015). The participation of GSH in the control of amino acid level was demonstrated in transgenic poplar with increased GSH level where 11 amino acids were detected in greater, 2 in smaller and 2 in similar, concentration in the transgenic lines compared with the wild type plants (Noctor 1998). In the present experimental system, similarly to the latter two studies, the redox treatment, except for the synthetic reductant DTT, resulted in greater amount of 27 amino acids. The restoration of the GSH level similar to that of Col-0 control (6-fold increase compared to the untreated mutant) in *pad2-1* mutant by exogenous GSH was accompanied by a 3-fold increase in total amino acid concentration (deriving from the considerable increase in the amount of nearly all individual amino acid) compared to the control Col-0. After the GSH treatment, very large changes in the ratios of the various amino acid families were detected in all 3 genotypes. The ratio of other amino acids (His and non-proteinogenic amino acids) increased from a maximum 5% to 40% with a parallel decrease in the total ratio of the aspartate and glutamate family from 80 to 50%. Although the level of many individual amino acids increased in the first family, the most determining one among them was the huge elevation in the concentration of alpha-aminoadipic acid (increase from 5–10% to 85–90%) indicating the special sensitivity of its metabolism to GSH. It is an analogue of Glu and is involved in the Lys metabolism. It inhibits Glu transport in animal systems (Haugstad and Langmoen 1997), but the conformation of such physiological role in plants needs future experiments. The present study indicates that most of the investigated 9 non-proteinogenic amino acids were greatly affected by redox treatments, which may indicate their important regulatory role. Unfortunately, in previous experiments only a few of them (Aaa, Gaba, Orn) were investigated regarding the possible redox control of their levels (Noctor 1998, Dumont et al. 2014, Noctor et al. 2015).

In contrast to the family of other amino acids, the ratio of serine family was not affected by GSH, but it was increased by the other treatments compared to the control plants in every genotype. It is surprising, that the ratio of this family was not influenced by GSH since two precursors of GSH (Cys and Gly) belong

to this family.

The results of correlation analysis corroborate the proposed redox control of amino acid levels. This analysis indicates a very strong effect of glutathione based on the correlations in pad2-1 mutants and GSH-treated plants.

Redox control of the amino acid levels in glutamate family

Although the ratio of the glutamate family was not affected by the various treatments except for GSH, special attention should be given to this family. It had a 50% ratio except for GSH treatment and, in general, the amount of the amino acid belonging to this family (Pro, Glu, Gln, Arg) was greatly increased by all the applied components of the AsA-GSH cycle but not by DTT. Glu is a central compound among amino acids since N from nitrate is incorporated into this amino acid. Also it is a common precursor of Pro, GSH and biogenic amines participating in the control of growth, development and stress response. The recently described redox sensitivity of NADH-dependent glutamine synthetase–glutamate synthase enzymes (Gómez-Baena et al. 2015) indicate the relationship between redox regulation and amino acid metabolism, which was also observed in the present study. The involvement of Glu in the stress response shown by an increase in its level in various plant species (Yang et al. 2000, Kovács et al. 2010, Kovács et al. 2011, Boldizsár et al. 2013) and the stress-induced changes in the concentration of AsA and GSH in wheat (Soltész et al. 2013) indicate, together with the present findings, a possible mediator role of AsA and GSH in the control of Glu content. In addition, according to recent studies, Glu can be a signaling molecule as observed in the roots based on its effect on root morphology and the presence of glutamate receptor proteins (Forde 2014). A cross-talk between redox and Glu-dependent signaling is also probable. A possible participant of this interaction can be α-aminoadipic acid since it was described as an inhibitor of Glu transport in animal system (Haugstad and Langmoen 1997) and its ratio was greatly increased by GSH in the present study.

Within the glutamate family the greatest changes occurred after the various treatments in Pro ratio. The increase in Pro is often accompanied by a reduction in Glu or Gln ratios due to their utilization in Pro synthesis. The very sensitive response of Pro to the redox treatments can be explained by its role in the maintenance of the redox homeostasis in plants (Szabados and Savouré 2010). Presumably there is a redox-based cross-talk between proline metabolism and AsA-GSH cycle (Anjum et al. 2014). This interaction exists, at least partly, at transcriptional level since many genes encoding enzymes of Pro metabolism were affected by the added components of the AsA-GSH cycle. Interestingly, both of the genes involved in Pro synthesis and in degradation were up-regulated, which may indicate a fine regulation of the two processes at the transcriptional level. In addition, the level of Orn involved in the *OAT* gene-related pathway of Pro synthesis was increased by GSH and H_2O_2 in *pad2-1* mutant and by

AsA in *vtc2-1* mutant. This intermediary compound of Pro synthesis can also modulate abiotic stress response as observed in *Arabidopsis* (Kalamaki et al. 2009). It was described previously that the *P5CS* and *OAT* pathways of Pro biosynthesis are not activated simultaneously by H_2O_2 (Yang et al. 2009), however some components of both pathways were induced by H_2O_2 in the present study. The role of *OAT* in redox homeostasis was also shown in rice since overexpression of *OAT* enhanced tolerance to oxidative stress because of the induction of Pro synthesis (You et al. 2012). However, the detected parallel increase in glutathione content and glutathione peroxidase activity can also contribute to the improvement of stress tolerance in this experiment. In animal system, *OAT* interacts with other cellular redox proteins such as glutaredoxin, thioredoxin, and it is regulated by *S*-glutathionylation (Liang et al. 2013). Based on the induction of *OAT* gene by GSH in all 3 *Arabidopsis* genotypes, such relationship can also be presumed in plants. Regarding the whole Pro metabolism, its special regulation by the AsA-GSH cycle can be suggested since, as at the metabolite level, the expression levels of the related genes were not affected by DTT except for *P5CS2* gene in *pad2-1* mutant and *OAT* in *vtc2-1* mutant.

Similarly to the amino acid levels, a very close correlation between the studied redox parameters and the components of the Pro metabolism was only observed in pad2-1 mutant, but not in the other two genotypes. These results indicate that the redox control of Pro metabolism exists at gene expression levels, which can be supplemented by the regulation at translational and enzyme activity levels. Similar changes in the expression of genes and activity of enzymes related to Pro metabolism can be supposed in the present experimental system based on previous studies in which relationship was found between Pro concentrations and levels of the corresponding transcripts and enzymes in salt-stressed artichoke and rape (Huang et al. 2013, Kubala et al. 2015).

Conclusions

The mutations and treatments modified the pool size and the redox state of ascorbate and glutathione, which, in turn, affected both the free amino acid composition and levels. The redox state of GSH was much strongly influenced because of its smaller pool size, which can be also a reason for the greatest effect of GSH treatment on the ratio and amount of free amino acids. GSH may also influence the activity of enzymes involved in the amino acid metabolism by (de)glutathionylation and thiol/disulphide conversion. The joint regulation of GSH and amino acid levels is possible through Glu, which is a common precursor of GSH and the amino acids in glutamate family. A further relationship is possible through alpha amino adipate, the level of which was highly increased by GSH treatment. This amino acid was described as an inhibitor of Glu transport in animal systems, however, this role should be confirmed in plants in future experiments. AsA and GSH may be special regulators of amino acid concentrations since they were not changed by the synthetic reductant, DTT. This statement is also supported by the

observed increase in the expression of the Pro metabolism-related genes following the AsA, GSH and H_2O_2 treatments.

Author contributions

G.Z. cultivated and treated the plants, L. S.-S. and Z. M. analyzed free amino acids, G. S. and G.Z. measured ascorbic acid and glutathione contents, G. Z. and E. B. investigated the gene expression levels, A. N. made the statistical analysis, G. Z., L. S.-S., G. G. and G. K. planned the experiments and prepared the manuscript.

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References

Anjum NA, Aref IM, Duarte AC, Pereira E, Ahmad I, Iqbal M (2014) Glutathione and proline can coordinately make plants withstand the joint attack of metal(loid) and salinity stresses. Front Plant Sci 5: 662

Bartosz G (1997) Oxidative stress in plants. Acta Physiol Plant 19: 47–64

Bell EA (2003) Nonprotein Amino Acids of Plants: Significance in Medicine, Nutrition, and Agriculture. J Agric Food Chem 51: 2854–2865

Benstein RM, Ludewig K, Wulfert S, Wittek S, Gigolashvili T, Frerigmann H, Gierth M, Flügge U-I, Krueger S (2013) Arabidopsis phosphoglycerate dehydrogenase1 of the phosphoserine pathway is essential for development and required for ammonium assimilation and tryptophan biosynthesis. Plant Cell 25: 5011–5029

Boldizsár A, Simon-Sarkadi L, Szirtes K, Soltész A, Szalai G, Keyster M, Ludidi N, Galiba G, Kocsy G (2013) Nitric oxide affects salt-induced changes in free amino acid levels in maize. J Plant Physiol 170: 1020–1027

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Chung PM, Cappel RE, Gilbert HF (1991) Inhibition of glutathione disulfide reductase by glutathione. Arch Biochem Biophys 288: 48–53

Conklin PL, Saracco SA, Norris SR, Last RL (2000) Identification of ascorbic acid-deficient Arabidopsis thaliana mutants. Genetics 154: 847–856

Considine MJ, Foyer CH (2014) Redox regulation of plant development. Antioxid Redox Signal 21: 1305–1326

Dietz K-J (2008) Redox signal integration: from stimulus to networks and genes. Physiol Plant 133: 459–

468

Dubreuil-Maurizi C, Vitecek J, Marty L, Branciard L, Frettinger P, Wendehenne D, Meyer AJ, Mauch F, Poinssot B (2011) Glutathione Deficiency of the Arabidopsis Mutant pad2-1 Affects Oxidative Stress-Related Events, Defense Gene Expression, and the Hypersensitive Response. Plant Physiol 157: 2000–2012

Dumont J, Keski-Saari S, Keinänen M, Cohen D, Ningre N, Kontunen-Soppela S, Baldet P, Gibon Y, Dizengremel P, Vaultier M-N, Jolivet Y, Oksanen E, Le Thiec D (2014) Ozone affects ascorbate and glutathione biosynthesis as well as amino acid contents in three Euramerican poplar genotypes. Tree Physiol 34: 253–266

Forde BG (2014) Glutamate signalling in roots. J Exp Bot 65: 779–787

Forde BG, Lea PJ (2007) Glutamate in plants: metabolism, regulation, and signalling. J Exp Bot 58: 2339–2358

Foyer CH, Noctor G (2011) Ascorbate and glutathione: the heart of the redox hub. Plant Physiol 155: 2–18

Geigenberger P, Fernie AR (2014) Metabolic control of redox and redox control of metabolism in plants. Antioxid Redox Signal 21: 1389–1421

Gómez-Baena G, Domínguez-Martín MA, Donaldson RP, García-Fernández JM, Diez J (2015) Glutamine Synthetase Sensitivity to Oxidative Modification during Nutrient Starvation in *Prochlorococcus marinus* PCC 9511. PLoS One 10: e0135322

Gulyás Z, Boldizsár A, Novák A, Szalai G, Pál M, Galiba G, Kocsy G (2014) Central role of the flowering repressor ZCCT2 in the redox control of freezing tolerance and the initial development of flower primordia in wheat. BMC Plant Biol 14: 91

Haugstad TS, Langmoen IA (1997) L-alpha-aminoadipate reduces glutamate release from brain tissue exposed to combined oxygen and glucose deprivation. J Cereb Blood Flow Metab 17: 567–570

Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A (2012) Role of proline under changing environments: A review. Plant Signal Behav 7: 1456–1466

Ho C-L, Saito K (2001) Molecular biology of the plastidic phosphorylated serine biosynthetic pathway in *Arabidopsis thaliana*. Amino Acids 20: 243–259

Huang Z, Zhao L, Chen D, Liang M, Liu Z, Shao H, Long X (2013) Salt Stress Encourages Proline Accumulation by Regulating Proline Biosynthesis and Degradation in Jerusalem Artichoke Plantlets. PLoS One 8: e62085

Kalamaki MS, Merkouropoulos G, Kanellis AK (2009) Can ornithine accumulation modulate abiotic stress tolerance in Arabidopsis? Plant Signal Behav 4: 1099–1101

Kellos T, Tímár I, Szilágyi V, Szalai G, Galiba G, Kocsy G (2008) Stress hormones and abiotic stresses

have different effects on antioxidants in maize lines with different sensitivity. Plant Biol Stuttg Ger 10: 563–572

Kocsy G, Laurie R, Szalai G, Szilagyi V, Simon-Sarkadi L, Galiba G, de Ronde JA (2005) Genetic manipulation of proline levels affects antioxidants in soybean subjected to simultaneous drought and heat stresses. Physiol Plant 124: 227–235

Kocsy G, Szalai G, Vágújfalvi A, Stéhli L, Orosz G, Galiba G (2000) Genetic study of glutathione accumulation during cold hardening in wheat. Planta 210: 295–301

Kocsy G, Tari I, Vanková R, Zechmann B, Gulyás Z, Poór P, Galiba G (2013) Redox control of plant growth and development. Plant Sci Int J Exp Plant Biol 211: 77–91

Kovács Z, Simon-Sarkadi L, Sovány C, Kirsch K, Galiba G, Kocsy G (2011) Differential effects of cold acclimation and abscisic acid on free amino acid composition in wheat. Plant Sci Int J Exp Plant Biol 180: 61–68

Kovács Z, Simon-Sarkadi L, Szucs A, Kocsy G (2010) Differential effects of cold, osmotic stress and abscisic acid on polyamine accumulation in wheat. Amino Acids 38: 623–631

Kranner I, Grill D (1996) Determination of Glutathione and Glutathione Disulphide in Lichens: a Comparison of Frequently Used Methods. Phytochem Anal 7: 24–28

Kubala S, Wojtyla Ł, Quinet M, Lechowska K, Lutts S, Garnczarska M (2015) Enhanced expression of the proline synthesis gene P5CSA in relation to seed osmopriming improvement of *Brassica napus* germination under salinity stress. J Plant Physiol 183: 1–12

Liang X, Zhang L, Natarajan SK, Becker DF (2013) Proline Mechanisms of Stress Survival. Antioxid Redox Signal 19: 998–1011

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(–Delta Delta C(T)) Method. Methods 25: 402–408

Martinelli T, Whittaker A, Bochicchio A, Vazzana C, Suzuki A, Masclaux-Daubresse C (2007) Amino acid pattern and glutamate metabolism during dehydration stress in the "resurrection" plant *Sporobolus stapfianus*: a comparison between desiccation-sensitive and desiccation-tolerant leaves. J Exp Bot 58: 3037–3046

Müller-Moulé P, Golan T, Niyogi KK (2004) Ascorbate-deficient mutants of Arabidopsis grow in high light despite chronic photooxidative stress. Plant Physiol 134: 1163–1172

Noctor G (1998) Manipulation of Glutathione and Amino Acid Biosynthesis in the Chloroplast. Plant Physiol 118: 471–482

Noctor G, Lelarge-Trouverie C, Mhamdi A (2015) The metabolomics of oxidative stress. Phytochemistry 112: 33–53

Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, Marquez-Garcia B, Queval G, Foyer CH (2012)

Glutathione in plants: an integrated overview. Plant Cell Environ 35: 454–484

Parisy V, Poinssot B, Owsianowski L, Buchala A, Glazebrook J, Mauch F (2007) Identification of PAD2 as a gamma-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of Arabidopsis. Plant J Cell Mol Biol 49: 159–172

Ros R, Muñoz-Bertomeu J, Krueger S (2014) Serine in plants: biosynthesis, metabolism, and functions. Trends Plant Sci 19: 564–569

Shaikhali J, Heiber I, Seidel T, Ströher E, Hiltscher H, Birkmann S, Dietz K-J, Baier M (2008) The redox-sensitive transcription factor Rap2.4a controls nuclear expression of 2-Cys peroxiredoxin A and other chloroplast antioxidant enzymes. BMC Plant Biol 8: 48

Soltész A, Smedley M, Vashegyi I, Galiba G, Harwood W, Vágújfalvi A (2013) Transgenic barley lines prove the involvement of TaCBF14 and TaCBF15 in the cold acclimation process and in frost tolerance. J Exp Bot 64: 1849–1862

Soltész A, Tímár I, Vashegyi I, Tóth B, Kellos T, Szalai G, Vágújfalvi A, Kocsy G, Galiba G (2011) Redox changes during cold acclimation affect freezing tolerance but not the vegetative/reproductive transition of the shoot apex in wheat. Plant Biol Stuttg Ger 13: 757–766

Suzuki N, Koussevitzky S, Mittler R, Miller G (2012) ROS and redox signalling in the response of plants to abiotic stress. Plant Cell Environ 35: 259–270

Szabados L, Savouré A (2010) Proline: a multifunctional amino acid. Trends Plant Sci 15: 89–97

Trovato M, Mattioli R, Costantino P (2008) Multiple roles of proline in plant stress tolerance and development. Rend Fis Acc Lincei 19: 325–346

Verbruggen N, Hermans C (2008) Proline accumulation in plants: A review. Amino Acids 35: 753–759

Vranova V, Rejsek K, Skene KR, Formanek P (2010) Non-protein amino acids: plant, soil and ecosystem interactions. Plant Soil 342: 31–48

Wang H, Wang J, Jiang J, Chen S, Guan Z, Liao Y, Chen F (2014) Reference genes for normalizing transcription in diploid and tetraploid Arabidopsis. Sci Rep 4: 6781

Yang C-W, Lin CC, Kao CH (2000) Proline, Ornithine, Arginine and Glutamic Acid Contents in Detached Rice Leaves. Biol Plant 43: 305–307

Yang S-L, Lan S-S, Gong M (2009) Hydrogen peroxide-induced proline and metabolic pathway of its accumulation in maize seedlings. J Plant Physiol 166: 1694–1699

You J, Hu H, Xiong L (2012) An ornithine δ-aminotransferase gene OsOAT confers drought and oxidative stress tolerance in rice. Plant Sci Int J Exp Plant Biol 197: 59–69

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Figure legends

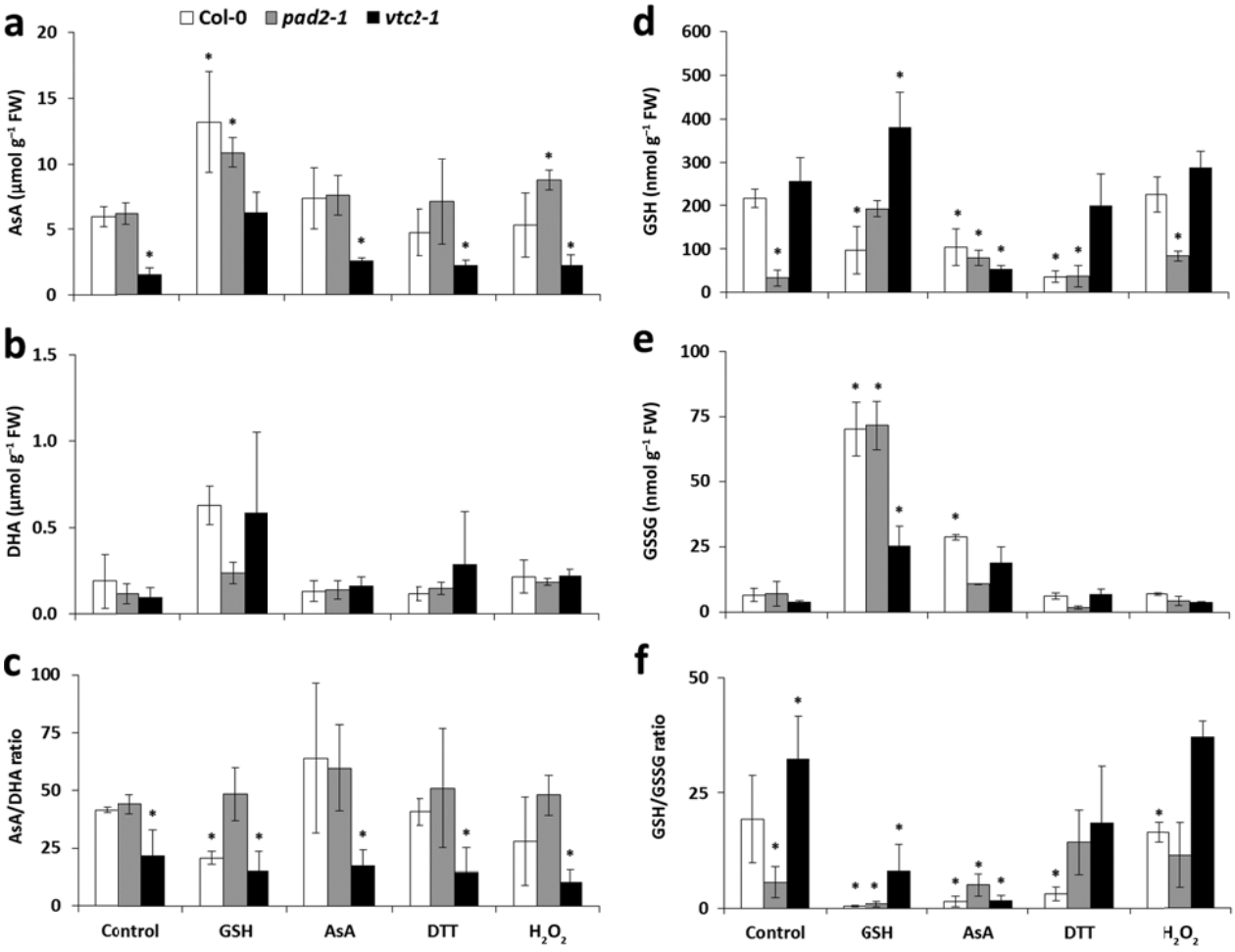

Fig. 1. Effect of redox treatments on glutathione and ascorbate content and their redox state. Redox changes were monitored by the measurement of AsA (a), DHA (b), GSH (d), GSSG (e) and the ratios of reduced and oxidized forms (c, f). 5-week old seedlings of Col-0 (wild type), *vtc2-1* (ascorbate-deficient mutant) and *pad2-1* (glutathione-deficient mutant) were treated by various reductants in 4 mM concentration (GSH: glutathione, AsA: ascorbic acid, DTT: dithiotreitol) and an oxidant, hydrogen-peroxide (H_2O_2), in 10 mM concentration for 3 days. Values indicated by asterisks are significantly different from the control Col-0, treated with no chemicals, at the P≤0.05% level.

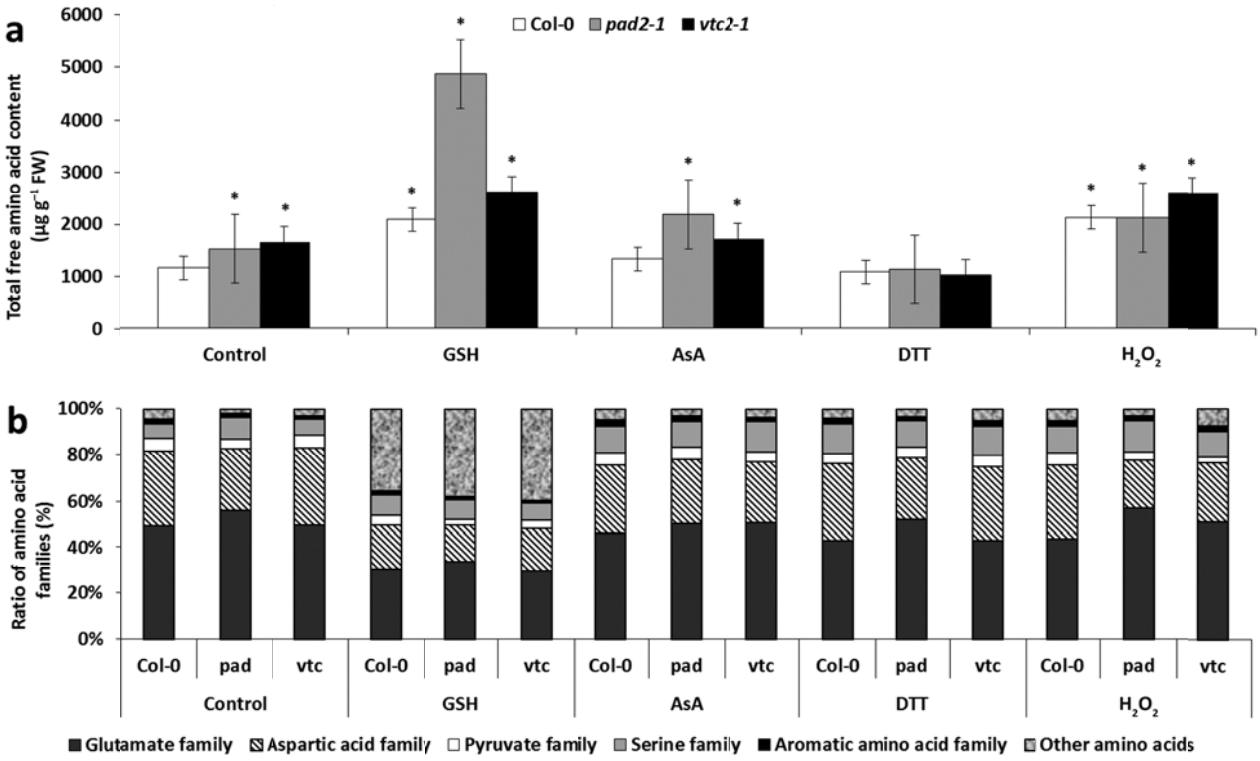

Fig. 2. Changes in the total free amino acid content (a) and the ratio of amino acid families (b). The experimental conditions are described in the legend of Fig. 1. Values indicated by asterisks are significantly different from the Col-0, treated with no chemicals, at the P≤0.05% level.

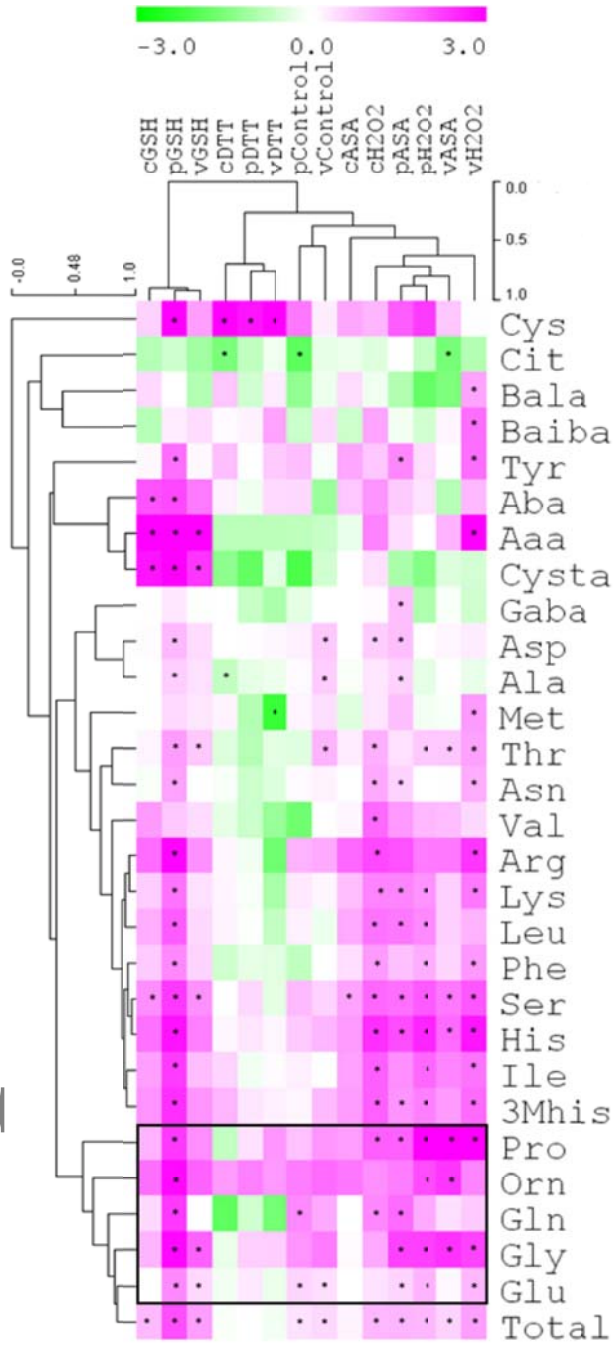

Fig. 3. Effect of the redox treatments on the relative levels of the free amino acids in the investigated genotypes (c: Col-0, p: *pad2-1*, v: *vtc2-1*). All values are normalized to the data detected in control Col-0 plants. Decreases and increases are shown on green and magenta backgrounds. The experimental conditions are described in the legend of Fig. 1. Non-proteogenic amino acids: Cit: citrulline; Bala: beta-aminoproprionic acid; Baiba: 3-aminoisobutyric acid; Aba: alpha-aminobutyric acid; Gaba: gamma-aminobutyric acid; Orn: ornithine; Aaa: alpha-aminoadipic acid; Cysta: cystathione. Values indicated by asterisks are significantly different from the corresponding control of Col-0, treated with no chemicals, at

the P≤0.05% level.

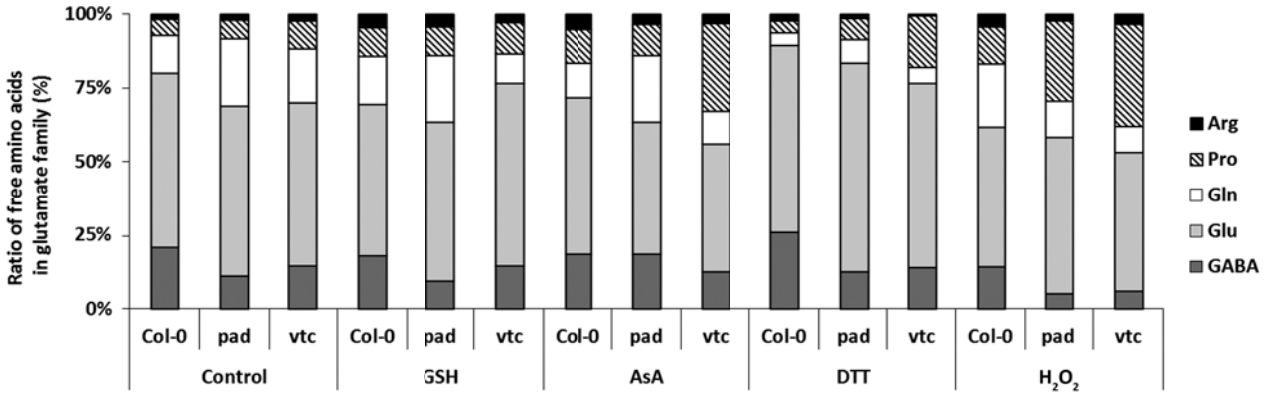

Fig. 4. Changes in the ratio of free amino acids in serine family. The experimental conditions are described in the legend of Fig. 1.

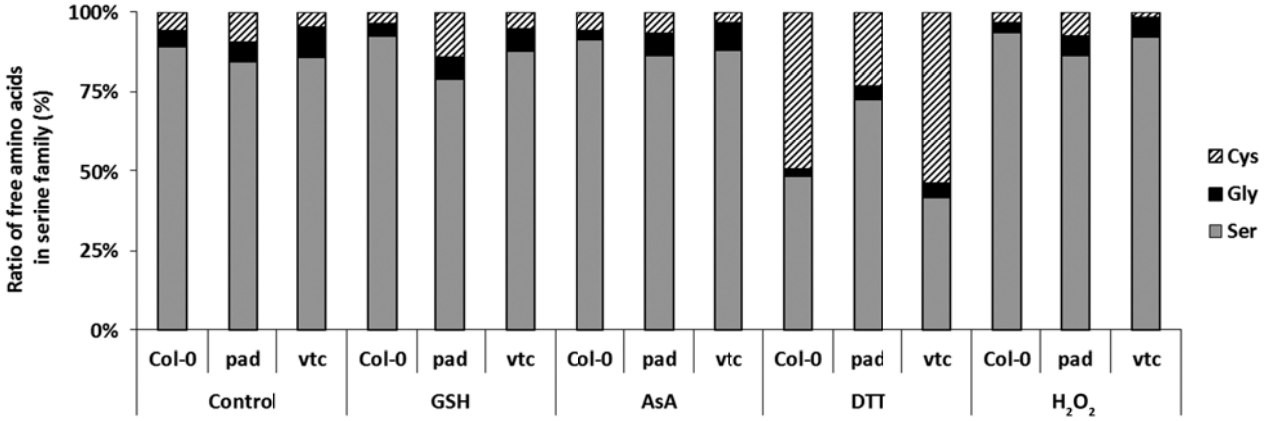

Fig. 5. Changes in the ratio of free amino acids in glutamate family. The ratio of Orn is not represented due to its too low level (1–2%). The experimental conditions are described in the legend of Fig. 1.

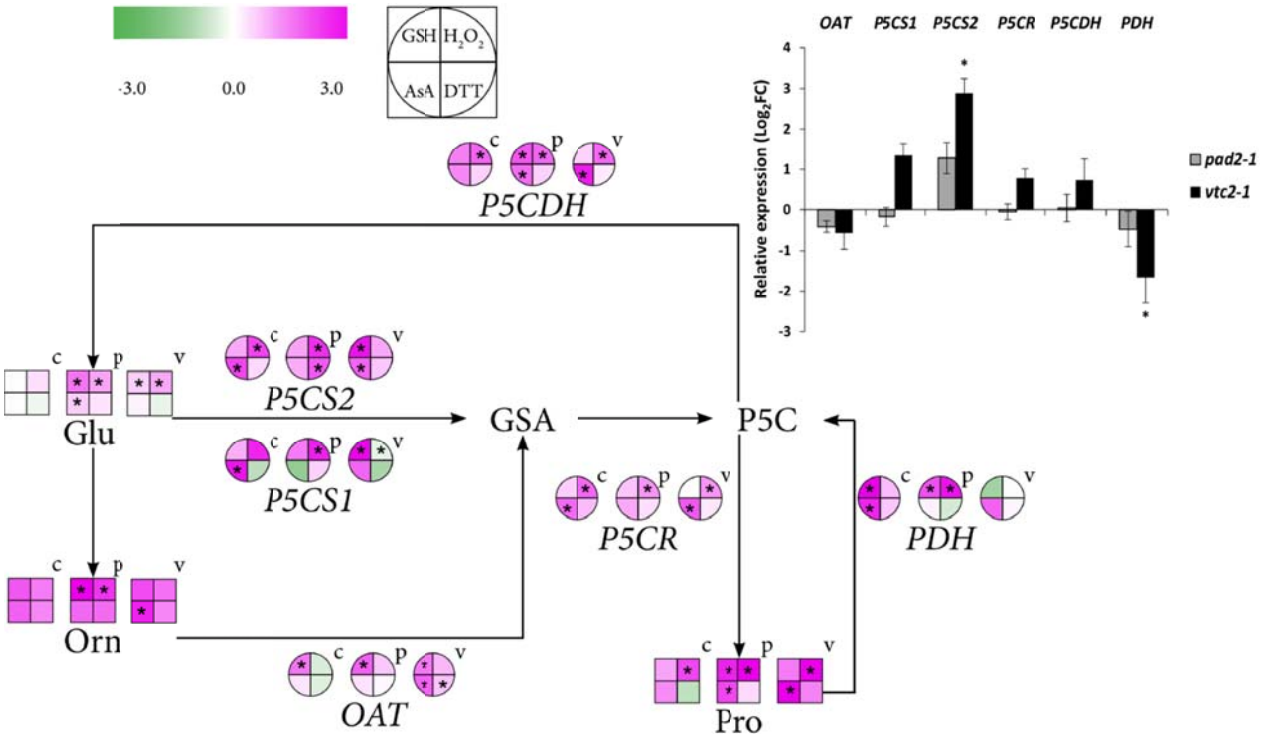

Fig. 6. The relative expression level of the genes involved in proline metabolism. The expression of ornithine aminotransferase (OAT), P5CDH (delta-1-pyrroline-5-carboxylate dehydrogenase), P5CS1 (delta 1-pyrroline-5-carboxylate synthase 1), P5CS2 (delta 1-pyrroline-5-carboxylate synthase 2), P5CR (pyrroline-5-carboxylate reductase) and PDH (proline dehydrogenase) and the level of Glu, ornithine (Orn) and Pro were detected after 3 days redox treatments described in the legend of Fig. 1. The expression levels are normalized to the control Col-0 for each genotypes and the metabolite contents to the own control of each genotypes. Values indicated by asterisks are significantly different from the corresponding controls, at the P≤0.05% level.