Acta Biologica Szegediensis

Volume 62(2):115-122, 2018
Acta Biologica Szegediensis
http://abs.bibl.u-szeged.hu/index.php/abs
DOI:10.14232/abs.2018.2.115-122

ARTICLE

Exogenous ascorbic acid is a pro-nitrant in *Arabidopsis thaliana*

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ABSTRACT Due to the intensified production of reactive nitrogen species (RNS) proteins can be modified by tyrosine nitration (PTN). Examination of PTN is a hot topic of plant biology, especially because the exact outcome of this modification is still pending. Both RNS and ascorbic acid (AsA) are redox-active molecules, which directly affect the redox state of cells. The possible link between RNS-dependent PTN and AsA metabolism was studied in RNS (*gsnor1-3*, *nia1nia2*) and AsA (*vtc2-3*) homeostasis *Arabidopsis* mutants. During physiological conditions, intensified PTN was detected in all mutant lines compared to the wild-type (WT); without altering nitration pattern. Moreover, the increased PTN seemed to be associated with endogenous peroxynitrite ($ONOO^-$) levels, but it showed no tight correlation with endogenous levels of nitric-oxide (NO) or AsA. Exogenous AsA caused intensified PTN in WT, *vtc2-3* and *nia1nia2*. In the background of increased PTN, significant NO and $ONOO^-$ accumulation was detected, indicating exogenous AsA-induced RNS burst. Interestingly, in AsA-triggered stress-situation, changes of NO levels seem to be primarily connected to the development of PTN. Our results point out for the first time that similarly to human and animal systems exogenous AsA exerts pro-nitrant effect on plant proteome. **Acta Biol Szeged 62(2):115-122 (2018)**

KEY WORDS
Arabidopsis
exogenous ascorbic acid
protein nitration
pro-nitrant

ARTICLE INFORMATION
Submitted
24 October 2018.
Accepted
15 November 2018.
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Introduction

On the analogy of oxidative stress, the concept of nitrosative stress became widely accepted in the last years. The group of molecules responsible for nitrosative stress - called reactive nitrogen species (RNS) – contains nitric oxide (NO), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), S-nitrosoglutathione (GSNO), S-nitrosothiols (RSNO), nitrogen dioxide (NO_2) or nitrosonium cation (NO^+) (Wang et al. 2013). In order to fulfil their role, they might co-interact with different signal molecules (e.g., MAPK cascade, cGMP, Ca^{2+}), or they are also able to directly modify proteins, fatty acids and presumably nucleic acids (Patel et al. 1999).

Tyrosine nitration, a posttranslational modification of proteins means the addition of a nitro group ($-NO_2$) to one of the two equivalent *ortho* carbons in the aromatic ring of the tyrosine amino acids (Gow et al. 2004). In this process, $ONOO^-$ plays an important role as the precursor of molecules chemically responsible for PTN itself (Yeo et al. 2015; Radi 2012). Peroxynitrite is formed in the reaction between superoxide anion ($O_2^{\cdot-}$) and NO at the production sites of $O_2^{\cdot-}$ (Denicola et al. 1998). PTN might affect the function and fate of a protein in different ways: beside no effect on the function (Begara-Morales et al. 2015), in most cases PTN results in the inhibition of protein activity (Greenacre and Ischiropoulos 2001; Radi 2004).

The result of PTN is mostly examined in stressed plants, in connection with the appearance of nitro-oxidative stress (Corpas et al. 2007; Mata-Pérez et al. 2016). Beyond stress-induced nitration, evidences suggest that PTN might happen during physiological conditions as well, which means that a part of the proteome is being nitrated even under control circumstances (reviewed by Kolbert et al. 2017). Furthermore, most of the results are obtained in *Arabidopsis* and crop plants, while we still have very little knowledge about the nitroproteome of mutant *Arabidopsis* lines.

Generation and different impacts of reactive oxygen species (ROS) dates back to the formation of oxygen-rich environment. High levels of ROS have the ability to damage macromolecules; hereby their concentration needs to be strictly controlled by the complex mechanisms of enzymatic- and non-enzymatic antioxidant systems (Apel and Hirt 2004). One of the most important non-enzymatic antioxidants is ascorbic acid (AsA) which is able to directly scavenge some of the ROS ($O_2^{\cdot-}$, singlet oxygen, hydroxyl radical, hydrogen peroxide (H_2O_2)) (Padh 1990); while through the activity of ascorbate peroxidase (APX) it participates indirectly in the elimination of H_2O_2 as well

(Foyer and Halliwell 1976; Asada 1992).

In plants exposed to environmental stresses, exogenous application of AsA has positive effects (Athar et al. 2009, Chao and Khao 2010). On the other hand, there is only limited data available about the effect of external AsA on healthy, unstressed plants and these studies reported pro-oxidant effects of exogenously applied AsA (Tyburski et al. 2012; Qian et al. 2014). Similarly, in human system, exogenously applied antioxidants, like AsA were shown to possess pro-oxidant property but besides, pro-nitrant effects have also been described (Bouayed and Bohn 2010).

The main goal of this study was to investigate the possible – but so far unknown – pro-nitrant (PTN-inducing) effect of exogenously applied AsA in a plant system. Also, the poorly known connection between physiological PTN and endogenous AsA levels has been examined using mutant *Arabidopsis thaliana* lines.

Materials and methods

Plant material and growth conditions

During the experiments, fourteen-day-old wild-type (WT, Col-0) and mutant *Arabidopsis thaliana* L. plants were used.

The *gsnor1-3* plants possess reduced S-nitrosoglutathione reductase (GSNOR) activity and higher total S-nitrosothiol, nitrate and NO levels (Feechan et al. 2005; Rustérucci et al. 2007; Lee et al. 2008). The *nia1nia2* mutant has a point mutation in NIA1 and a deletion in NIA2 gene, having only 0.5% of the nitrate reductase (NR) enzyme activity of the WT (Wilkinson and Crawford 1993). The *vtc2-3* contains 40-50% of the WT AsA level, caused by a mutation in VTC2 gene, responsible for GDP-L-galactose phosphorylase synthesis (Conklin 2001). All *Arabidopsis* lines had *Columbia* (*Col*) ecotype background.

The seeds of all plant lines were surface sterilised with 70% (v/v) ethanol followed by 5% (v/v) sodium hypochlorite and transferred to half-strength Murashige and Skoog medium (1% (w/v) sucrose and 0.8% (w/v) agar) (Murashige and Skoog 1962). In case of external AsA supply (100 and 500 μM), autoclaved agar medium was cooled to approximately 35 °C before the addition of AsA in order to avoid heat-caused degradation. Moreover, the pH of the medium was adjusted to 7 instead of the normal 5.7-5.8, to avoid its acidification after AsA supplementation.

The petri dishes were kept in a greenhouse at a photo flux density of 150 μmol m^{-2}/s (12/12 day/night period) at a relative humidity of 55-60% and 25 ± 2 °C.

Determination of AsA

250 mg plant material was grounded in 1 ml 5% (w/v) trichloroacetic acid (TCA) during sample preparation. The amount of total ascorbate was determined by the reduction of dehydroascorbate to ascorbate by dithiothreitol (DTT); the reduced AsA samples contained water instead of DTT. Ascorbate concentrations were determined spectrophotometrically at 525 nm and are expressed in μmol/g fresh weight. Dehydroascorbate content was calculated as the difference between total and reduced AsA concentration (Law et al. 1983).

Detection of NO, $ONOO^-$ and $O_2^{\cdot-}$ by fluorescent microscopy

For the detection of NO 1,2-diaminoanthraquinone (DAQ) was used (Seligman et al. 2008). Seedlings were incubated in 50 μM DAQ solution prepared in ultrapure water for 30 min at room temperature followed by a single washing step in water prior microscopic analysis. As control experiment NO donor sodium nitroprusside (SNP) was used (200 μM), while as NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; 800 μM) was applied; both SNP and cPTIO were applied for one hour at 150 μmol m^{-2}/s light intensity.

The content of $ONOO^-$ in the root tips was detected using dihydrorhodamine (DHR) staining (Sarkar et al. 2014). Roots were incubated in 10 μM DHR solution prepared in Tris-HCl (10 mM, pH 7.4), and were washed twice with Tris-HCl buffer. For testing the response of DHR to $ONOO^-$ and H_2O_2, *Arabidopsis* plants were pretreated with peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1; 1000 μM; 1 h) or H_2O_2 (100 μM; 30 min).

Dihydroethidium (DHE) was used for visualisation of superoxide anion. Root tips were incubated in 10 μM dye solution for 30 min in darkness at 37 °C and were washed twice with TRIS-HCl buffer (10 mM, pH 7.4) (Kolbert et al. 2012).

The root tips of *Arabidopsis* plants stained with different fluorophores were investigated under a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany) equipped with a high resolution digital camera (Axiocam HR) and filter set 9 (exc.: 450-490 nm; em.: 515- ∞ nm) for DHE, filter set 10 (exc.: 450-490; em.: 515-565 nm) for DHR, filter set 20HE (exc.: 546/12; em.: 607/80) for DAQ. Fluorescence intensities (pixel intensity) in the meristematic zones of the primary roots were measured on digital images using Axiovision Rel. 4.8 software within circles of 100 μm radii.

Preparation of protein extract, SDS-PAGE and western blotting

Plant material was grounded with double volume of extraction buffer (50 mM Tris-HCl buffer, pH 7.6-7.8, containing 0.1 mM EDTA (ethylene diamine tetra acetic acid), 0,1% Triton X-100 (polyethyleneglycol p-(1,1,3,3-tetra-methylbutyl)-phenylether) and 10% glycerol. After

20 min centrifugation on 4 °C at 12 000 rpm the supernatant was stored at -20 °C. Protein concentration was determined using the Bradford assay (Bradford 1976) with bovine serum albumin as standard.

Protein extracts (30 μg per lane) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gels. For western blot analysis, separated proteins were transferred to PVDF membranes using the wet blotting procedure (30 mA, 16 h). After transfer, membranes were used for cross-reactivity assays with rabbit polyclonal antibody against 3-nitrotyrosine diluted 1:2000 (Corpas et al. 2008). Immunodetection was performed by using affinity isolated goat anti-rabbit IgG-alkaline phosphatase secondary antibody in dilution of 1:10 000, and bands were visualised by using NBT/BCIP reaction. As a positive control nitrated bovine serum albumin (NO_2-BSA) was used.

Statistics

The results are expressed as mean ± SE. Multiple comparison analyses were performed with SigmaStat 12 software using analysis of variance (ANOVA, $P<0.05$) and Duncan's test. All experiments were carried out at least two times; in each treatment, at least 10 samples were measured.

Results and discussion

Nitrosative status of non-stressed Arabidopsis lines

Compared to the WT, *vtc2-3* line – in agreement with the literature (Conklin et al. 2000; Conklin 2001) – contained lower, 52% of the wild-type total AsA. Interestingly, the NO overproducer *gsnor1-3* had extremely high AsA level (almost twice as much as the WT), compared to the other lines (Fig. 1A). In this mutant, the majority of the glutathione pool is S-nitrosylated and de-nitrosylation of the GSNO is decreased because of the lower GSNOR activity (Feechan et al. 2005). In the absence of reduced glutathione, the ascorbate-glutathione cycle cannot work properly, which may lead to *de novo* AsA biosynthesis (Colville and Smirnoff 2008). It must be mentioned that there was no statistically significant difference between the oxidised AsA content of the different lines.

In order to check the NO or $ONOO^-$ dependence of the applied fluorophores, control experiments were conducted. The inducing effect of NO-donor (SNP) and the decreasing effect of NO scavenger (cPTIO) on DAQ fluorescence (Fig. 2A) together suggest that DAQ fluorescence detects NO in *Arabidopsis* tissues. Further results indicate (Fig. 2B) that DHR detects $ONOO^-$ but not H_2O_2.

Gsnor1-3 root tips showed 76% higher NO level those of in the WT (Fig. 1B), possibly because of the low GSNOR activity and the consequently high GSNO content serving as NO source or reservoir (Lindermayr et al. 2005). Additionally, the NO and $ONOO^-$ levels in the *vtc2-3* root tips proved to be significantly elevated compared to the wild-type (Fig. 1B and 1C).

Endogenous AsA content of *nia1nia2* line was similar

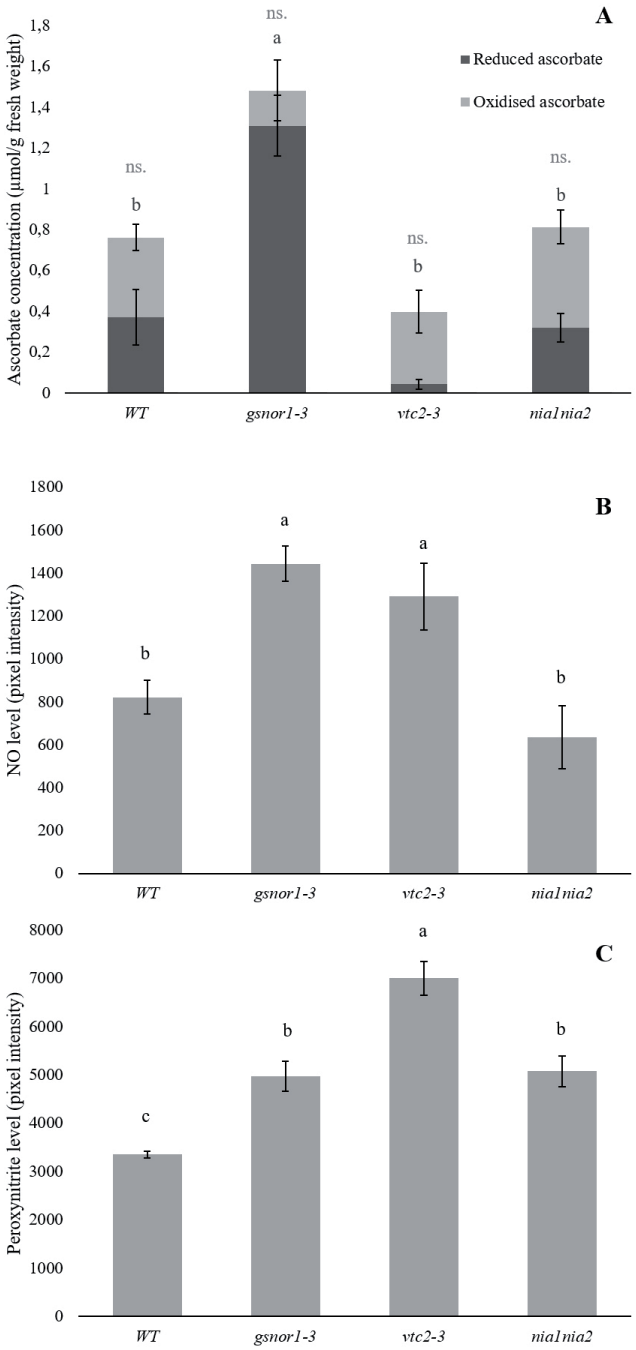

Figure 1. AsA (**A**), NO (**B**) and $ONOO^-$ (**C**) levels in 14-days-old WT and mutant *Arabidopsis* lines under control conditions. The lack of significance (n.s.) or the different letters indicate significant differences according to Duncan-test (n = 10, P≤0.05).

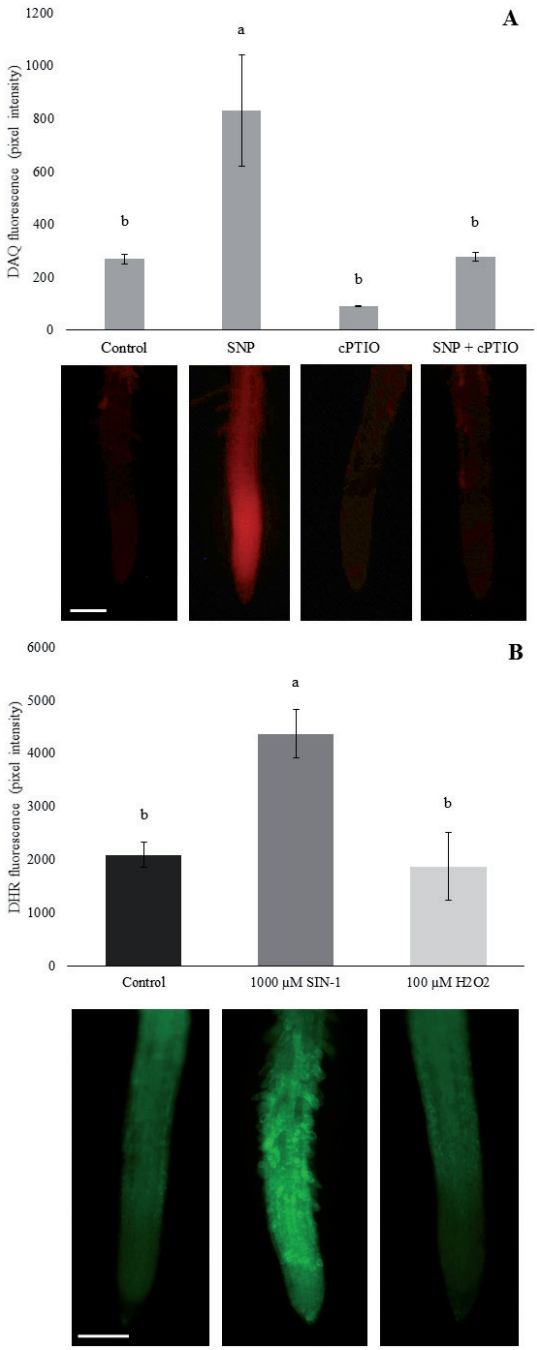

Figure 2. Fluorescent intensities and representative pictures of DAQ-stained *Arabidopsis* roots pre-treated with NO donor and/or scavenger (A), and DHR-stained *Arabidopsis* roots after peroxynitrite donor or H_2O_2 treatment (B). NO donor SNP (200 µM) and scavenger cPTIO (800 µM) were applied for one hour at 150 µmol m^{-2} s^{-1} light intensity; peroxynitrite donor SIN-1 was applied for 1 h (1000 µM) and H_2O_2 for 30 min (100 µM). Different letters indicate significant differences according to Duncan-test (n = 10, P≤0.05). Bar = 1 mm.

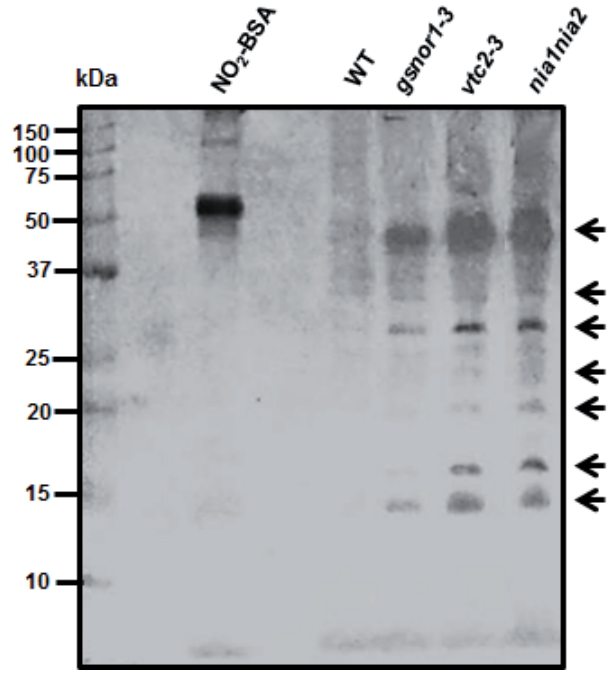

Figure 3. Representative immunoblot showing protein tyrosine nitration in 14-days-old WT and mutant *Arabidopsis* plants under control conditions. As a positive control nitrated bovine serum albumin (NO_2-BSA) was used. Arrows indicate nitrated protein lanes.

to the WT (Fig. 1A) and this line – in agreement with previous results (Pető et al. 2011) - showed lower NO level (77%) in its root tips relative to the WT (Fig. 1B). This is most likely caused by the lower activity of NR (Wilkinson and Crawford 1993), the main NO source in the roots (Chamizo-Ampudia et al. 2017). Compared to the WT, in *nia1nia2*, significantly elevated $ONOO^-$ levels were detected (Fig. 1C), which might be the result of the reaction between NO and superoxide anion. This may be supported by the previously published high superoxide radical level in *nia1nia2* roots (Pető et al. 2011).

The detectable PTN, even during control circumstances, is in accordance with previously published results (Chaki et al. 2015; Tanou et al. 2012) indicating the occurrence of physiological nitroproteome in unstressed plants. Moreover, PTN was intensified in all mutant lines compared to the WT which suggests that a bigger proportion of the proteome suffers nitration due to mutations. Interestingly, the pattern of nitration was the same in all plant lines; raising the possibility that similar proteins might become nitrated. Consequently, results show that different mutations affected the frequency of PTN, but it did not influence its pattern.

In case of *gsnor1-3,* high NO, $ONOO^-$ and AsA contents were accompanied by slightly intensified PTN compared to the WT. In contrast, the proteome of *vtc2-3* showing relatively low AsA level, but notably elevated NO and $ONOO^-$ content proved to be intensively tyrosine ni-

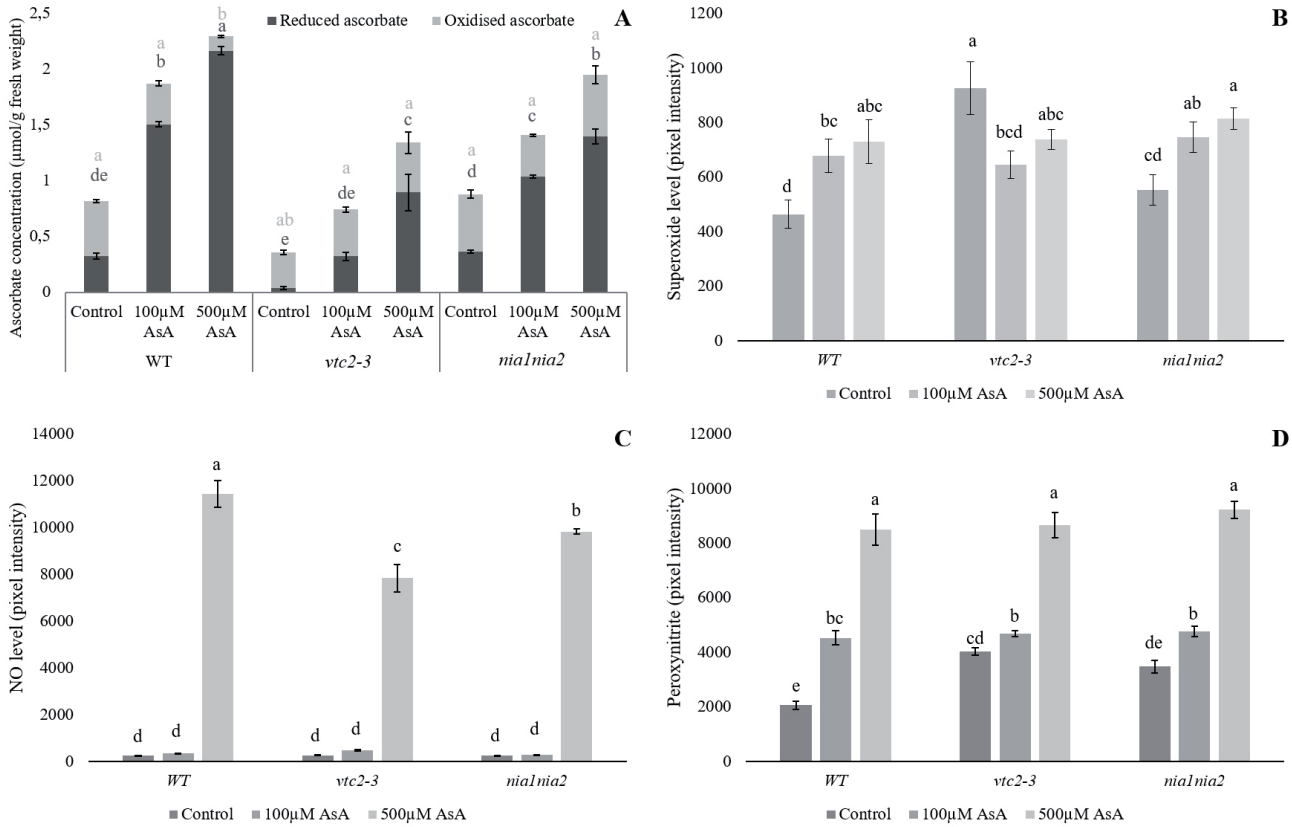

Figure 4. AsA (A), NO (B) $O_2^{\bullet-}$ (C) and $ONOO^-$ (D) levels in 14 days-old WT and mutant *Arabidopsis* lines after two weeks of exogenous AsA treatment. Different letters indicate significant differences according to Duncan-test (n = 10, P≤0.05).

trated. In NO underproducer *nia1nia2*, intensified PTN was detected, while $ONOO^-$ levels were enhanced and AsA content was WT-like. These comparisons point out that physiological protein tyrosine nitration has no tight correlation with endogenous NO content of the plant tissues. At the same time, PTN showed a positive correlation with $ONOO^-$ levels suggesting that the rate of protein nitration is associated with the tissue level of $ONOO^-$ being the source molecule of direct nitrating agents ($NO_2^{\bullet}$ and $CO_3^{\bullet-}$; Souza et al. 2008). Moreover, we found no clear relationship between endogenous AsA levels and physiological protein tyrosine nitration in *Arabidopsis* (Fig. 3).

Exogenous AsA induces PTN

Our further experiments with NO underproducer *nia1nia2* and AsA deficient *vtc2-3* lines intended to answer the question whether exogenous AsA could revert the increased PTN of these plants or AsA rather exerts pro-nitrant effect similarly to animal systems.

Significant differences were observed between the AsA accumulation properties of the *Arabidopsis* lines (Fig. 4A). Wild-type plants accumulated the most AsA in absolute value, reaching 2.29 µmol total ascorbate per one-gram fresh weight, and most of the AsA was present in the reduced form. In *vtc2-3,* 500 µM AsA treatment resulted in almost 4-fold increase in total AsA content, which is significantly larger relative increase than in the WT (3-fold). Interestingly, the AsA uptake did not decrease the quantity of the oxidised form in this case. The relative increase of AsA values in *nia1nia* was similar to WT; however, in absolute values it accumulated less AsA (Fig. 4A).

The $O_2^{\bullet-}$ content increased significantly in WT and *nia1nia2* lines as the effect of both 100 and 500 µM AsA treatment, while in *vtc2-3,* 100 µM AsA significantly decreased $O_2^{\bullet-}$ level (Fig. 4B). In *vtc2-3*, the reduced endogenous AsA content resulted in higher $O_2^{\bullet-}$ level, which might be reverted by external AsA supplementation. Despite the AsA-induced $O_2^{\bullet-}$ accumulation, there was no significant increase in lipid peroxidation (data not shown), suggesting that the externally applied AsA at these concentrations did not cause remarkable oxidative stress. In the work of Qian et al. (2014), exogenous AsA exerted pro-oxidant effect on *Arabidopsis* seedlings, although the concentrations were remarkably higher (2 mM or 8 mM) than in our experiments.

The NO content of the root tips increased significantly as the effect of the highest applied concentration, where we detected a sharp increase in NO levels in all three lines (Fig. 4C). Exogenously applied AsA induced NO accumulation also in the nitrate reductase-deficient *nia1nia2* mutant indicating that this enzyme is not involved in NO biosynthesis triggered by AsA. Rather non-enzymatic mechanisms may contribute to NO accumulation like the AsA-regulated reduction of nitrite at acidic pH (Crawford 2006) and/or the AsA-induced decomposition of GSNO-reservoirs (Kashiba-Iwatsuki et al. 1996).

The $ONOO^-$ levels of the root tips were significantly increased by exogenous AsA as well (Fig. 4D). In absolute values, all lines accumulated similar amount of $ONOO^-$ after 500 μM AsA treatment; however, in terms of relative accumulation the plant lines differed. In WT, 100 and 500 μM AsA caused 2- and 4-fold increase respectively, while in *vtc2-3,* we measured only 1.1- and 2-fold; in *nia1nia2* 1.3- and 2.5-fold increase in $ONOO^-$ levels compared to control. It should be noted, that unlike NO, $ONOO^-$ content was significantly increased by 100 μM AsA treatment as well; and in case of *vtc2-3* $ONOO^-$ level in control root tips were significantly higher than in WT. These indicate that exogenous AsA induces NO and $ONOO^-$ (representing RNS) burst in *Arabidopsis* root tips.

Then it is not surprising that 100 and 500 μM AsA significantly increased PTN in all three examined *Arabidopsis* lines. The degree of nitration was the highest in WT, but it increased remarkably also in the mutant lines (Fig. 5).

Unlike in the control experiment, the differences in the intensity of the nitration showed correlation with the NO levels, but not with the $ONOO^-$ content in case of 500 μM AsA treatment. Moreover, the most intense PTN was accompanied by the highest NO level in WT. The similar PTN levels in 100 μM AsA-treated plants seem to be connected with NO levels, as well as with $ONOO^-$ contents. Furthermore, the pattern of nitration changed compared to the control experiments, however the different AsA concentrations did not affect PTN pattern.

Exogenous AsA did not ameliorate nitrosative modification of *Arabidopsis* proteome, but it exerted a remarkable pro-nitrant effect. Moreover, the exogenous AsA-induced PTN seems to be more associated with NO level than with that of $ONOO^-$.

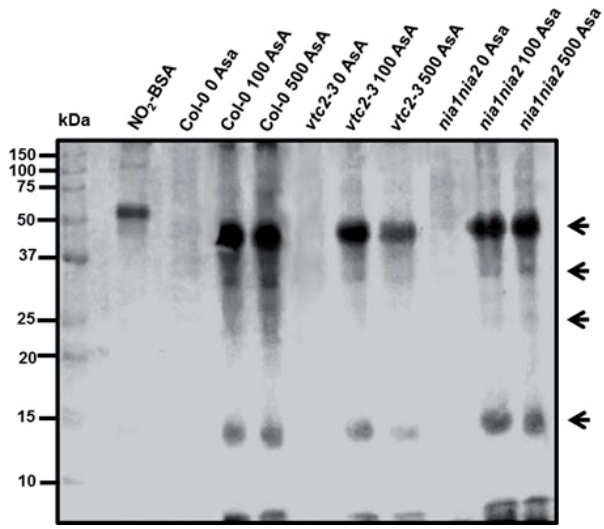

Figure 5. Representative immunoblot showing protein tyrosine nitration in WT and mutant *Arabidopsis* plants after two weeks of exogenous AsA treatment (100 or 500 μM). As a positive control nitrated bovine serum albumin (NO_2-BSA) was used. Arrows indicate nitrated protein lanes.

Conclusions

According to our knowledge, this is the first study investigating PTN in RNS/AsA metabolism mutant *Arabidopsis* under control conditions and during AsA supplementation. Data clearly show that physiological PTN in non-stressed plants is associated with endogenous peroxynitrite but not with NO levels. Furthermore, there is no correlation between the size of the endogenous AsA pool and the size of the physiological nitroproteome in *Arabidopsis*.

Applied together with abiotic stressors, AsA acts as an antioxidant (Athar et al. 2009; Chao and Kao 2010), however, its effect on healthy, non-stressed plants has been poorly studied. The limited amount of data describes the pro-oxidant and growth-reducing effect of exogenous AsA (Tyburski et al. 2012; Qian et al. 2014). In the background of the pro-oxidant effect of AsA, Qian et al. (2014) discovered the downregulation of antioxidant enzymes. This downregulation however can also be caused by protein tyrosine nitration processes, described in the present study. Thus – as a feedback loop – the failure in the antioxidant system might increase ROS accumulation, leading to the further intensification of PTN. Our results support that exogenous AsA at the applied concentrations acts as a stressor, causing RNS burst and subsequent PTN, thus it has pro-nitrant property. Interestingly, in this AsA-induced stress-situation, NO seems to be primarily connected to the development of PTN. Exogenous ascorbic acid as a pro-nitrant has been known in humans and animals for a while (Bouayed and Bohn 2010), but this is the first plant study to prove the pro-nitrant effect of this originally antioxidant molecule applied exogenously; however further research is needed to clarify the exact mechanism behind this phenomenon.

Acknowledgements

This work was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (BO/00751/16/8) and by the National Research, Development and Innovation Fund (Grants no. NKFI-6, K120383 and NKFI-1, PD120962). Zs. K. was supported by UNKP-17-4 New National Excellence Program of the Ministry of Human Capacities.

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