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 peroxyxynitrite (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.

KEY WORDS
- Arabidopsis
- exogenous ascorbic acid
- protein nitration
- pro-nitrant

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 (N₂O₃), S-nitrosogluthathione (GSNO), S-nitrosothiols (RSNO), nitrogen dioxide (NO₂) 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, Ca²⁺), 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 (-NO₂) 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 (O₂⁻) and NO at the production sites of O₂⁻ (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 (O₂•-, singlet oxygen, hydroxyl radical, hydrogen peroxide (H₂O₂)) (Padh 1990); while through the activity of ascorbate peroxidase (APX) it participates indirectly in the elimination of H₂O₂ as well.
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 with double volume of extraction buffer (50 mM Tris-HCl buffer, pH 7.6-7.8, containing 0.1 mM EDTA (ethylenediamine 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 (NO₂-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 H₂O₂.

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.
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-
treated. In NO underproducer \textit{nia1nia2}, intensified PTN was detected, while ONOO\textsuperscript{-} 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\textsuperscript{-} levels suggesting that the rate of protein nitration is associated with the tissue level of ONOO\textsuperscript{-} being the source molecule of direct nitrating agents (NO\textsubscript{2}\textsuperscript{-} and CO\textsubscript{3}\textsuperscript{2-}; Souza et al. 2008). Moreover, we found no clear relationship between endogenous AsA levels and physiological protein tyrosine nitration in \textit{Arabidopsis} (Fig. 3).

**Exogenous AsA induces PTN**

Our further experiments with NO underproducer \textit{nia1nia2} and AsA deficient \textit{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 \textit{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 \textit{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 \textit{nia1nia2} was similar to WT; however, in absolute values it accumulated less AsA (Fig. 4A).

The O\textsubscript{2}\textsuperscript{.} content increased significantly in WT and \textit{vtc2-3} lines as the effect of both 100 and 500 µM AsA treatment, while in \textit{vtc2-3}, 100 µM AsA significantly decreased O\textsubscript{2}\textsuperscript{.} level (Fig. 4B). In \textit{vtc2-3}, the reduced endogenous AsA content resulted in higher O\textsubscript{2}\textsuperscript{.} level, which might be reverted by external AsA supplementation. Despite the AsA-induced O\textsubscript{2}\textsuperscript{.} 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 \textit{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·.

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.
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References


