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ORIGINAL ARTICLE

Rapid ascorbate response to bacterial elicitor treatment in *Arabidopsis thaliana* cells

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Abstract An early event of the incompatible plant–pathogen interactions is an oxidative burst. On one hand, the ROS generated during oxidative burst is advantageous. ROS can serve as secondary messengers mediating defence gene activation and establishment of additional defences. On the other hand, the concentration of ROS must be carefully regulated to avoid undesired cellular cytotoxicity. The major water soluble, low molecular weight antioxidant, ascorbic acid plays a crucial role in ROS balancing (scavenging). The regulation of ascorbate level, therefore, can be an important point of the fine-tuning of ROS level during the early phase of plant–pathogen interaction. To evaluate how this interaction affects the biosynthesis, the recycling, and the level of ascorbate, we challenged *Arabidopsis thaliana* cells with two different harpin proteins ($HrpZ_{pto}$ and $HrpW_{pto}$). $HrpZ_{pto}$ and $HrpW_{pto}$ treatments caused a well-defined ROS peak. The expression of the alternative oxidase (AOX1a) and vtc5, one of the paralog genes that encode the rate limiting enzyme of ascorbate biosynthesis, followed the elevation of ROS. Similarly, the activity of ascorbate peroxidase and galactono-1,4-lactone dehydrogenase (EC 1.3.2.3) (GLDH), the enzyme catalysing the ultimate, mitochondria coupled step of ascorbate biosynthesis and the level of ascorbate and glutathione also followed the elevation of ROS due to harpin treatment. The enhanced expression of AOX1a, the elevated activity of GLDH, and the increased level of ascorbate and glutathione all can contribute to the mitigation or absence of programmed cell death. Finally, a new function, the fine-tuning of redox balance during plant–pathogen interaction, can be proposed to vtc5.

Keywords Oxidative burst · ROS · Harpin proteins · Ascorbate · Glutathione · Hypersensitive response · Plant–pathogen interaction

Introduction

An early event of the incompatible plant–pathogen interactions is an oxidative burst (Torres 2010). The activation of plasma membrane-localized NADPH-oxidases (Mur et al. 2008), cell wall peroxidases (Bindschedler et al. 2006; Choi et al. 2007), and apoplastic amine, diamine, and polyamine oxidases (Allan and Fluhr 1997) is involved in the increase of reactive oxygen species (ROS). Different subcellular organelles, such as the mitochondria (Garmier et al. 2007; Cvetkovska and Vanlerberghe 2013), chloroplasts, and peroxisomes (Karpinski et al. 2003; Camejo et al. 2016), also take part in the generation of ROS. This oxidative burst response in incompatible interactions consists of two distinct phases (Baker et al. 1991; Keppler 1989). The nonspecific phase I occurs directly after the addition of either compatible or incompatible pathogens; however, phase II is a relatively long-lasting response. It occurs 1.5–3 h after inoculation and it seems to be limited only to incompatible pathogens (Baker and Orlandi 1995).

On one hand, the ROS generated during oxidative burst are advantageous. The extremely reactive species, such as

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the hydroxyl radical, can kill the pathogen in a direct way (Chen and Schopfer 1999). They can also take part in the construction of physical barriers (Bradley et al. 1992; Hückelhoven 2007). ROS can behave as secondary messengers mediating defence gene activation and initiate of additional defences through the redox control of transcription factors and establish the interaction with other signalling pathways, such as phosphorylation cascades (Kovtun et al. 2000; Mou et al. 2003; Kuźniak 2010). ROS can also generate jasmonate-type signalling cyclic oxylipins (Montillet et al. 2005) and phytoalexins, secondary metabolites to arrest pathogen growth (Thoma et al. 2003). Last but not least, ROS are also associated with the hypersensitive response (HR), a form of programmed cell death localized at the site of pathogen attack that can contribute to prevent the growth of pathogens (Mur et al. 2008). HR has an important role in the development of a plant immune response and it is often associated with the systemic acquired resistance (SAR) (Feys and Parker 2000). Harpins are type-three secretion system delivered proteins from Gram-negative plant pathogen bacteria. During the plant–pathogen interaction, these proteins are secreted into the intercellular space of plant tissue and trigger a HR in nonhost plants (Reboutier et al. 2007). Looking to the other side of the coin, it can be realized that the concentration of ROS must be carefully regulated to avoid undesirable cytotoxicity (Grant and Loake 2000). For the signalling role of ROS, nontoxic levels must be maintained by the fine-tuning of ROS production and ROS-scavenging pathways (Mittler et al. 2004).

The plant antioxidant system has both enzymatic and low molecular weight elements. The major water soluble, low molecular weight antioxidant, ascorbic acid plays a crucial role in the majority of ROS balancing (scavenging) complex antioxidant processes (Szarka et al. 2012). Ascorbate can directly reduce superoxide, singlet oxygen, and hydroxyl radicals, and it is also a substrate for ascorbate peroxidase (APX) that catalyses the conversion of hydrogen peroxide to water. Furthermore, the ascorbate–glutathione cycle is present in the cytosol, chloroplasts, mitochondria, apoplast, and peroxisomes (Chew et al. 2003; Szarka et al. 2012, 2013; Szarka 2013). Therefore, it is not surprising that low level of ascorbate equally reduces growth and increases susceptibility to a range of abiotic stresses (Szarka et al. 2012). Sensitivity to ozone and other abiotic stresses, such as freezing and UV-B irradiation, could be observed in the ascorbate-deficient vtc1 mutant *Arabidopsis* (Conklin et al. 1996). However, vtc1 and vtc2 mutation and the accompanying ascorbate deficiency resulted in increased level of salicylic acid and transcript levels of genes encoding pathogen-related (PR) proteins, increased resistance to virulent pathogens, elevated peroxidase activity, and PR gene transcript level, and, furthermore, the accumulation of the phytoalexin camalexin (Barth et al. 2004; Pavet et al. 2005; Colville and Smirnoff 2008; Kliebenstein 2004). All these results suggest that ascorbate deficiency augments plant biotic defence cascades leading to greater disease resistance. During plant–pathogen reactions, the activities and levels of the ROS detoxifying enzymes APX and catalase are suppressed by salicylic acid and NO (Klessig et al. 2000). The suppression of ROS detoxifying mechanisms is crucial for the onset of HR (Apel and Hirt 2004). All these observations underline the importance of the coordinated production of ROS and downregulation of ROS-scavenging mechanisms. Furthermore, ascorbate and glutathione are also signal-transducing molecules (Foyer and Noctor 2011).

The regulation of the level of ascorbate, therefore, must be one of the most important elements of the fine-tuning of ROS level during plant–pathogen interaction. Interestingly, nothing is known about the regulation of key elements of ascorbate biosynthesis, such as vtc2 and vtc5 (Dowdle et al. 2007; Szarka et al. 2012), and GLDH (Szarka et al. 2013), or recycling (members of the ascorbate–glutathione cycle) during plant–pathogen interaction. To evaluate how this interaction affects the biosynthesis, the recycling, and the level of ascorbate, we challenged *Arabidopsis thaliana* cells with two different harpin proteins ($HrpZ_{pto}$ and $HrpW_{pto}$) from *Pseudomonas syringae* pv tomato DC3000.

HrpZ and HrpW belong to the early described harpins of *Pseudomonas syringae* pv tomato DC3000 (Charkowski et al. 1998; Preston et al. 1995). HrpZ proteins from various *Pseudomonas syringae* pathovars bind lipids and form ion-conducting pores in liposomes and synthetic membrane bilayers (Lee et al. 2001). HrpZ also binds to plant plasma membrane originated vesicles, it inserts into the membrane, causing vesicle disruption (Haapalainen et al. 2011). HrpZ induces a strong hypersensitive response in tobacco leading to necrotic lesions (Haapalainen et al. 2012). HrpW consists of two domains. The N-terminal forms a harpin domain and the C-terminal is homologous to a PL3 pectate lyase (Coutinho and Henrissat 1999). The pectate lyase domain binds to calcium pectate beads, but enzymatic activity does not associate with it (Charkowski et al. 1998). Only the N-terminal harpin domain shows exogenous HR elicitation (Charkowski et al. 1998); however, HrpW homology is less strongly conserved in the harpin domain than in the pectate lyase domain (Chang et al. 2004). Both HrpZ and HrpW are conserved harpins of *Pseudomonas syringae* pathovars. Their expression rapidly increases on the induction of *hrp–hrc* genes (Ferreira et al. 2006; Lindeberg et al. 2006). Since both HrpZ and HrpW induce a large transient oxidative burst (Reboutier et al. 2007; Krause and Durner 2004), we used these two different bacterial harpin proteins ($HrpZ_{pto}$ and $HrpW_{pto}$) to study

how this interaction affects the biosynthesis, the recycling, and the level of ascorbate.

Materials and methods

Materials

Murashige and Skoog medium, 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin, 2-(*N*-morpholino)ethanesulfonic acid (MES), triphenil-tetrazolium chloride (TTC), xylenol orange, EDTA, 4-morpholinepropanesulfonic acid (MOPS), Polyvinylpyrrolidone (PVP-40), reduced and oxidized glutathione, hydroxylamine, sulphanilamide, α-naphthylamine, dehydroascorbate, and ampicillin were obtained from Sigma-Aldrich. Monochlorobimane (mBCl) and ProBond Purification System were purchased from Invitrogen. The Amicon Ultra 30 K Centrifugal Filter Units were purchased from Merck. L-galactono-1,4-lactone was from Carbosynth Ltd., UK. IPTG was obtained from Fermentas, cytochrome c came from Fluka, and ascorbic acid was bought from Riedel-de Häen. All other chemicals were of analytical or HPLC grade, and were purchased from Reanal, Hungary. Pierce Coomassie (Bradford) Protein Assay Kit, GeneJET Plant RNA Purification Kit, and RevertAid First-Strand cDNA Synthesis Kit were obtained from Thermo Scientific; SensiFAST SYBR No-ROX Kit came from Bioline.

Plant material

Arabidopsis thaliana (ecotype Columbia) suspension cells were grown in culture medium containing 0.44% MS + Gamborg (Sigma-Aldrich); 3% Sucrose; 0.24 µg/ml 2,4-dichlorophenoxyacetic acid; 0.014 µg/ml Kinetin; 4 mM PBS (K_2HPO_4, KH_2PO_4); pH 5.8 in a rotary shaker (120 rpm) at 22 °C, in the dark. The cells were subcultured weekly by a tenfold dilution.

Harpin production

The harpin producing *Escherichia coli* cells (Kvitko et al. 2007) were grown in 2 l Erlenmeyer flasks containing 500 ml LB-Amp medium (1% Bacto Tryptone; 0.5% Bacto Yeast-Extract; 0.5% NaCl; 50 µg/ml Ampicillin) in a rotary shaker (180 rpm) at 37 °C. The bacteria were incubated to an optical density at 600 nm (OD_{600}) of ca. 0.5. To induce harpin production, IPTG was added to a final concentration of 1 mM and the cultures were incubated for an additional 4 h at 32 °C. The cells were harvested (5300*g* for 10 min at 4 °C) and the pellets were stored at −20 °C until protein purification.

Harpin purification

The pellets were defrosted and suspended in 8 ml Guanidinium Lysis Buffer (6 M Guanidine-HCl; 500 mM NaCl; 20 mM Na_3PO_4, pH 7.8); then, the suspension was sonicated on ice for 6 × 10 s. The lysate was centrifuged (5300*g* 10 min) and the supernatant was gently harvested.

Harpin proteins were purified from the supernatant under hybrid conditions using *Invitrogen ProBond Purification System*, as described in the user manual. After elution, the buffer was changed to 5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.8) using Merck Millipore Amicon Ultra-2 ml 30 K (Kvitko et al. 2007). Purified harpins were stored at −20 °C until use.

The quality of the proteins was verified by SDS-PAGE, and the concentrations were determined by *Pierce Coomassie (Bradford) Protein Assay Kit*, using BSA solution (*bovine serum albumin*) as standards.

Harpin treatments

The experiments were conducted on 4-day-old *A. thaliana* cultures. The required volume of harpin preparation was added to the culture, while the control cells were treated with the same volume of 5 mM MES (pH 5.8) buffer. At the indicated time points, 10 ml of *Arabidopsis* cells were harvested by vacuum filtration and frozen in liquid nitrogen (Desikan et al. 2001).

Cell viability assay

Cell viability was determined by the slightly modified triphenil-tetrazolium chloride (TTC) reduction assay (Castro-concha et al. 2006). Briefly, 20 mg of TTC was dissolved in 1 ml 50 mM phosphate buffer (pH 7.5) and stored until use in the dark at 4 °C. A known amount of freshly vacuum filtrated *Arabidopsis* cells were transferred to a microfuge tube. Cells were washed with 50 mM phosphate buffer (pH 7.5) and re-suspended in 980 µl of the same buffer and supplemented with TTC stock solution to a final concentration of 1.25 mM. This mixture was incubated for 3 h in the dark. After the incubation, it was centrifuged (16,000*g*, 2 min), the supernatant was discarded, and 1.2 ml of ethanol was added to solubilize the formed formazan salts. After 8 h of incubation, the cells were centrifuged (16,000*g*, 2 min), and the absorbance of the supernatant was measured at 485 nm. Cell viability was normalized to the freshly harvested, vacuum filtrated cell weight.

Hydrogen peroxide determination

Hydrogen peroxide production was followed by xylenol orange assay (Wolff 1994). The reagent was prepared as

the following: 1 ml of solution A (25 mM $FeSO_4$, 25 mM $(NH_4)_2SO_4$, and 2.5 M H_2SO_4) was added to 100 ml of solution B (125 µM xylenol orange and 100 mM sorbitol). Because of their instability, the solutions were freshly prepared just before the assays (Bindschedler et al. 2001).

At different time points, 1 ml of *A. thaliana* suspension was withdrawn and centrifuged at 16,000×*g* for 1 min. 50 µl of the supernatant was added immediately to 950 µl xylenol orange reagent (1 ml solution A + 100 ml solution B), and the samples were incubated for 45 min at room temperature. The absorbance of the reaction mixture was measured at 560 nm against a blank, containing cell culture medium. The assay was calibrated using H_2O_2 standards. To ensure that the absorbance increase was caused by H_2O_2 formation, samples supplemented with 10 U catalase were also measured (Bindschedler et al. 2006).

Assay for superoxide anion

The detection of superoxide anion was carried out as described by Unger et al. (2005).

The method detects superoxide by the oxidation of hydroxylamine to nitrite. Briefly, at the indicated time points, 135 µl of *A. thaliana* suspension cells were withdrawn and incubated with 135 µl Na-phosphate buffer (50 mM, pH 7.8) and 30 µl hydroxylamine (10 mM) in the dark. After 45 min, the samples were centrifuged at 16,000×*g* for 2 min and 100 µl of the supernatant was transferred to a 96-well microtiter plate. To detect the nitrite content of the samples, 100 µl sulphanilamide (17 mM in 30% acetic acid) and 100 µl α-naphthylamine (7 mM in water) were added to each sample, and the absorbance was measured at 540 nm. The calibration curves were established for 0–100 µM NO_2^-.

From the reaction: $2O_2^{\cdot-} + H^+ + NH_2OH \rightarrow H_2O_2 + H_2O + NO_2^-$, the concentration of $O_2^{\cdot-}$ was calculated according to the following equation $2[O_2^{\cdot-}] = [NO_2^-]$. To verify that nitrite production was due to superoxide generated by the cells, a reaction mixture without hydroxylamine was used (Wu and Von Tiedemann 2001).

Isolation of mitochondria and GLDH activity measurement

Following the harpin treatment ca., 100 g of *Arabidopsis* cells were harvested by vacuum filtration and the cells were disrupted using a grinder. Mitochondria were isolated by differential centrifugation and Percoll density gradient centrifugation as described by Zsigmond et al. (2011).

L-galactono-1,4-lactone dehydrogenase (GLDH) activity was measured photometrically. The assay relies on the L-galactono-1,4-lactone dependent reduction of cytochrome c at room temperature. The reaction mixture consisted of cytochrome c (1.5 mg/ml), L-galactono-1,4-lactone (11.4 mM) and isolated mitochondria in 0.4 M mannitol, 10 mM MOPS, and 1 mM EGTA (pH 7.2), in a final volume of 1 ml. The reduction of cytochrome c was monitored by measuring the increase of absorbance at 550 nm. Under these conditions, the absorbance increase was proportional to the enzyme activity and was linear with respect to time for an initial period of 2 min (Oba et al. 1994; Zsigmond et al. 2011).

Gene expression analysis

RNA was isolated from the frozen *Arabidopsis* cells by *GeneJET Plant RNA Purification Kit*. First-strand cDNA synthesis for RT-PCR was done by *Thermo Scientific RevertAid First-Strand cDNA Synthesis Kit,* using $Oligo(dT)_{18}$ primer.

Real-time PCR was conducted with each primer pair listed below (Table 1), employing the following protocol: 95 °C/3 min, 30 cycles of 95 °C/30 s, and 60 °C/30 s by *Thermo Scientific PikoReal* real-time PCR system, using *SensiFAST SYBR No-ROX Kit*. The mitosis protein YLS8 was used as housekeeping gene (Remans et al. 2008).

Ascorbate–glutathione cycle assays

Freshly harvested, vacuum filtrated *A. thaliana* cells were frozen in liquid nitrogen and homogenized by mortar and pestle. The frozen powder was dissolved in 1 ml of extraction buffer [50 mM MES pH 6; 2 mM $CaCl_2$; 40 mM KCl; 1 mM ascorbate (freshly added)], and centrifuged (48,000×*g* for 20 min. at 4 °C). Activities of all enzymes of the cycle were measured from the supernatant.

Ascorbate peroxidase (APX) activities were measured by following the oxidation of ascorbate at 290 nm using an extinction coefficient of 2.8 mM^{-1} cm^{-1} in the presence of 1.5 mM ascorbate. The reaction was initiated by the addition of 0.1 M H_2O_2 to the reaction mixture (50 mM potassium-phosphate, pH 7). The nonspecific ascorbate oxidation was also measured as blank using the same conditions without adding cell lysate extract (Nakano and Asada 1981).

Monodehydroascorbate reductase (MDHAR) activity was assayed by the rate of decrease in absorbance at 340 nm because of the oxidation of NADH content of the reaction buffer (50 mM HEPES, 0.25 mM ascorbate, 0.25 mM NADH, pH 7.6). The substrate (MDHA) was generated by 2.5 U/ml ascorbate-oxidase (EC 1.10.3.3), added to each sample. The nonspecific NADH oxidation was also measured as blank using the same conditions without adding ascorbate-oxidase. The extinction coefficient used was 6.22 mM^{-1} cm^{-1} (Nakano and Asada 1981).

Table 1 List of primers that were used for real-time PCR quantification

Gene	Sequences	References
YLS8	fw: 5′-TTA CTG TTT CGG TTG TTC TCC ATT T-3′	Remans et al. (2008)
	rv: 5′-CAC TGA ATC ATG TTC GAA GCA AGT-3′	
AOX1a	fw: 5′-CCG ATT TGT TCT TCC AGA GG-3′	Rasmusson et al. (2004)
	rv: 5′-GCG CTC TCT CGT ACC ATT TC-3′	
GLDH	fw: 5′-CCC AGT GGA TGC ATA CAA CAA-3′	Designed by our group
	rv: 5′-GTG GTG GAG ACT GGG AAG AG-3′	
VTC2	fw: 5′-CAA TGT TAG TCC GAT AGA GTA TGG-3′	Zhang et al. (2009)
	rv: 5′-TGT AAC CGA GTC TGA AGT ATG G-3′	
VTC5	fw: 5′-AAT GTG AGT CCG ATT GAG TAT GG-3′	Zhang et al. (2009)
	rv: 5′-AGT AAG CCT GAA AGT GAA GAT GG-3′	

Dehydroascorbate reductase (DHAR) activity was determined by monitoring the rate of ascorbate formation at 265 nm using an extinction coefficient of 14 mM^{-1} - cm^{-1}. The reaction buffer consisted of 50 mM HEPES (pH 7), 0.1 mM EDTA, 25 mM GSH, and 1.2 mM dehydroascorbate. The nonspecific ascorbate formation was also measured as blank using the same conditions without adding cell lysate extract (Stahl et al. 1983).

Glutathione reductase (GR) activity was determined by measuring the decrease in absorbance at 340 nm due to NADPH oxidation. The reaction was initiated by adding 1 mM of GSSG to the reaction buffer (50 mM HEPES, 0.5 mM EDTA, 0.25 mM NADPH, pH 8). The nonspecific NADPH oxidation was also measured as blank, using the same conditions without adding GSSG. The extinction coefficient used for the calculations was 6.22 mM^{-1} cm^{-1} (Mannervik 2001).

Antioxidant determination

A known amount of freshly harvested, vacuum filtrated plant cells were homogenized on ice in 5% acetic acid for ascorbate determination or in 5% SSA (sulphosalicylic acid) for glutathione determination by Potter–Elvehjem homogenizer. The lysate was centrifuged (48,000*g*, 20 min, 4 °C) and the supernatant was gently harvested.

Ascorbate and dehydroascorbate levels were determined by reverse phase HPLC as described by (Balogh and Szarka 2016). For dehydroascorbate determination, 100 µl of the supernatant was mixed with 10 µl 50 mM DTT to reduce the dehydroascorbate content of the sample. The analyses were carried out using Thermo Finnigan Surveyor HPLC equipped with Teknokroma Mediterranea Sea18 (C18) 5 µm 15 × 0.46 column.

Determination of GSH content of the samples was performed as described earlier in (Zsigmond et al. 2011). The pH of 100 µl supernatant was adjusted to 7.8 by the addition of 12 µl 4 M TEA (triethanolamine). The GSH content of the solution was derivatized with 1 mM monochlorobimane (mBCl). The mBCl reacts specifically with thiol groups producing a highly fluorescent thioether. Separation of derivatized GSH was performed on a Teknokroma Nucleosil 100 C-18 column with 5 µm beads and dimensions of 4.6 × 250 mm, using Waters 2690 HPLC, followed by fluorescent detection (Waters 2475).

Both ascorbate and GSH content were normalized to the freshly harvested, vacuum filtrated cell weight, which was used for the Potter–Elvehjem homogenization.

Other methods

Protein concentration was determined using the BioRad protein assay solution with bovine serum albumin as a standard, according to the manufacturer's instructions. All data are expressed as means ± S.D. Statistical analyses (Student's *t* test) were performed with SPSS version 13.0.1 (SPSS Inc, Chicago, IL).

Results

$HrpZ_{pto}$ and $hrpW_{pto}$ provoked oxidative burst in Arabidopsis cells

In the first set of experiments, *Arabidopsis* cells were treated by two different harpin proteins from *P. syringae* pv. tomato DC3000 ($HrpZ_{pto}$, $HrpW_{pto}$), and then, the viability of the cells and the level of ROS generated due to the treatment were followed. Neither of the harpins had any influence to the viability of the cells in the applied concentration (150 nM) even at longer incubation times (6 or 24 h) (Fig. 1). At the same time, a well-defined oxidative burst could be observed. The generation rate of superoxide anion increased to 0.44 nmol/g FW/min (320% increase) and 0.54 nmol/g FW/min (412% increase) after 30 min of $HrpZ_{pto}$ and $HrpW_{pto}$ treatments, respectively (Fig. 2, panel a). The level of H_2O_2 increased to 6.5 µM (189% increase) and 8.5 µM (225% increase) after 60 min of $HrpZ_{pto}$ and

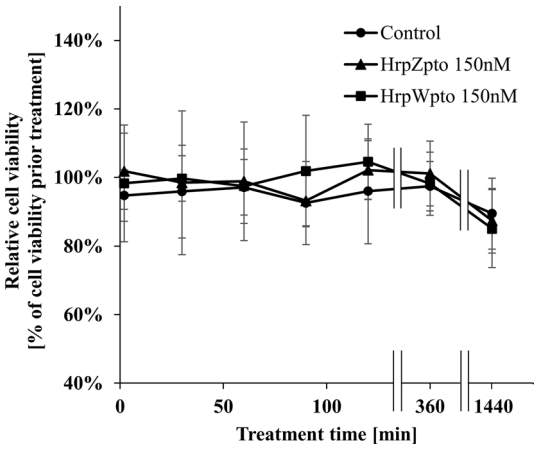

Fig. 1 Effect of $HrpW_{pto}$ and $HrpZ_{pto}$ treatment on cell viability of *Arabidopsis* suspension cells. *Arabidopsis* suspension cells were treated with harpin at 150 nM final concentration. At indicated time points, cell viability was estimated by TTC reduction assay as described in Materials and methods. The cell viability of the samples, those were collected from every cell culture before any treatment, was regarded as 100%. Value represents mean ± SD from three independent harpin treatments

$HrpW_{pto}$ treatment, respectively (Fig. 2, panel b). Following these peak values, the level of ROS descended to the control 120 min after the addition of harpins. It seems that the generation of superoxide anion is prior to the generation of hydrogen peroxide (Fig. 2, panel a, b). Although it is logical, since the action of superoxide dismutase resulted in the generation of H_2O_2, however, it should also be noted that the superoxide detection assay requires a 45 min incubation of the cells in the reaction mixture that allows the measurement of superoxide anion amount generated through the whole incubation period. This way, the acquired data rather represent a time interval than a well-defined time point. As it could be expected the elevation of ROS accompanied by the elevation of the mRNA level of the oxidative stress marker AOX1a (Fig. 3) (Polidoros et al. 2009). The time course of AOX1a expression followed that of the generation of ROS (Figs. 2, 3). All these observations suggest that our attempt to cause an oxidative burst in Arabidopsis cell culture by harpin treatment was successful.

The effect of harpin treatment on ascorbate biosynthesis

GDP-L-galactose phosphorylase is encoded by vtc2 and its paralogue vtc5. It catalyses the committed step of ascorbate biosynthesis (Dowdle et al. 2007; Urzica et al. 2012). This step is also considered to be the rate limiting, primarily regulated step of the pathway (Dowdle et al. 2007; Urzica et al. 2012). Thus, in the second turn of our experiments, the effect of harpin treatment was investigated on the expression of both genes. Interestingly, the expression of the two genes was differentially affected by the harpins. The mRNA level of vtc2 did not show any difference on the treatment (Fig. 4, panel a). However, the expression of vtc 5 started to increase after 60 min of elicitor treatment and reached its peak value at 90 min with 435–535% expression levels, and then, it descended (Fig. 4, panel b). Significant differences could not be observed between the effects of the two different harpin proteins (Fig. 4).

Although it is not likely that galactono-1,4-lactone dehydrogenase (GLDH) is the rate limiting step of ascorbate biosynthesis (Szarka et al. 2013), but its activity is regulated by the respiratory electron flow (Millar et al. 2003) and this must be speeded up in harpin-induced oxidative burst (Vidal et al. 2007). Thus, both the expression and the activity of GLDH were investigated. According to our assumption, the expression of GLDH did not change due to harpin treatment (Fig. 4, panel c), but its activity followed the level of ROS and AOX1a (Fig. 5).

The effect of harpin treatment on ascorbate recycling

The recycling of the oxidized forms of ascorbate is as important in the regulation of its level as its biosynthesis, and thus the effect of harpin treatments on the activities of the enzymes of ascorbate–glutathione cycle was also studied. The activity of APX and monodehydroascorbate reductase (MDHAR) was enhanced by $HrpZ_{pto}$ treatment. Both of them reached their maximal value after 90 min and then descended (Fig. 6, panel a, b). However, $HrpZ_{pto}$ did not have any effect on dehydroascorbate reductase (DHAR) and glutathione reductase (GR) activities (Fig. 6, panel c, d). Similarly, $HrpW_{pto}$ had no notable effect on any enzyme of the cycle (Fig. 6).

The effect of harpin treatment on the levels of ascorbate and glutathione

The summary of all above-investigated pathways is realized in the level of ascorbate in plant cells. Thus, finally, the level of ascorbate was also determined both in harpin-treated and nontreated plant cells. Both harpin treatment caused the elevation of ascorbate content by 35–50%. However, the time courses showed a little difference in the case of the two different harpin proteins. The total ascorbate content of $HrpZ_{pto}$-treated plant cells reached a local maximum at 30 min and then dropped back to the level of control at 60 min; finally, it was elevated again at 90 and 120 min (Fig. 7, panel a). The total ascorbate content of $HrpW_{pto}$-treated plant cells showed a 30 min delay compared to the $HrpZ_{pto}$ treated with a local maximum value at 60 min (Fig. 7, panel a). Both at 90 and 120 min of incubation time, both $HrpZ_{pto}$- and $HrpW_{pto}$-treated cells showed increased

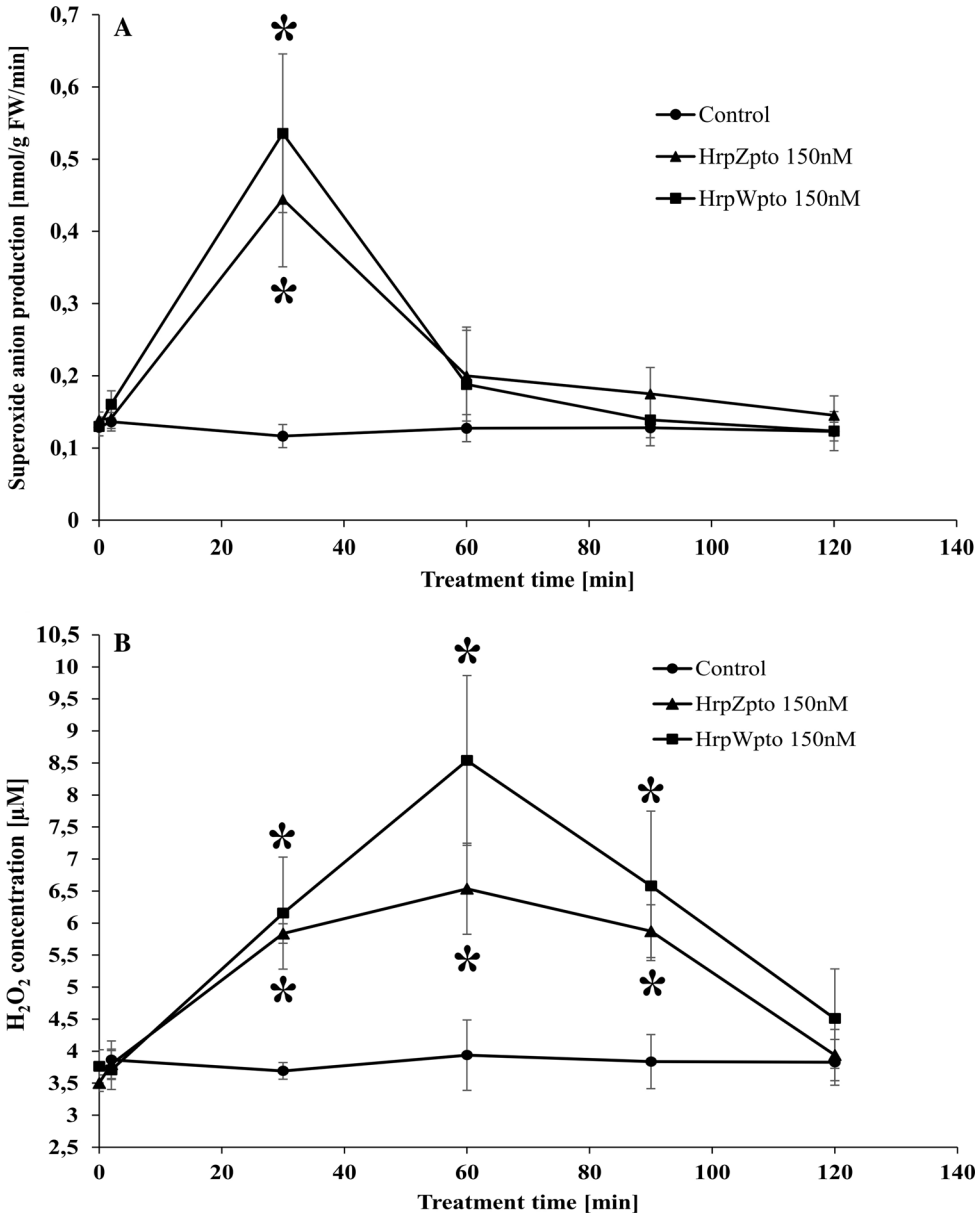

Fig. 2 Effect of $HrpW_{pto}$ and $HrpZ_{pto}$ treatment on ROS [superoxide anion (**a**) and H_2O_2 (**b**)] generation (level) of *Arabidopsis* suspension cells. *Arabidopsis* suspension cells were treated with $HrpW_{pto}$ and $HrpZ_{pto}$ at 150 nM final concentration. At indicated time points, samples were taken and the generation of superoxide anion was followed by the oxidation of hydroxylamine to nitrite, the H_2O_2 content was determined by xylenol orange assay as described in "Materials and methods". Value represents mean ± SD from three independent harpin treatments. (*Asterisk*) Significant difference with respect to control ($P < 0.01$)

total ascorbate contents (Fig. 7, panel a). The redox state of the ascorbate pool was also affected by the harpin treatment. Only a minor shift to the more oxidative side (approx. 4%) was caused by the $HrpZ_{pto}$ treatment (Fig. 7, panel b). However, a bit more marked oxidative shift (approx. 10% at 90 min) could be observed after the addition of $HrpW_{pto}$ (Fig. 7, panel a). This later one recovered to the redox state of $HrpZ_{pto}$-treated cells. The fluctuation of the redox state of the control samples was within 1%.

Similar to the ascorbate content, an elevated level of glutathione could be observed in the harpin-treated plant cells (Fig. 8, panel a). Both $HrpZ_{pto}$ and $HrpW_{pto}$ treatment caused a nonsignificant, but notable enhancement of cellular glutathione content with a maximum at 90 min. Similar to the time course pattern of ascorbate, the GSH content of $HrpZ_{pto}$-treated cells increased 30 min earlier than that of the $HrpW_{pto}$-treated cells (Fig. 8). However, there was no decrease (local maximum) in the glutathione content of $HrpZ_{pto}$-treated cells (Figs. 7, panel a vs 8, panel a). The glutathione level decreased only at the end of the investigated period (at 120 min) in both harpin-treated cell cultures. Furthermore, the redox state of glutathione was not influenced by either harpin treatments (Fig. 8, panel b).

Discussion

One of the earliest responses and typical hallmarks of plant–pathogen interaction is the induction of extreme ROS generation, the so-called oxidative burst (Apel and Hirt

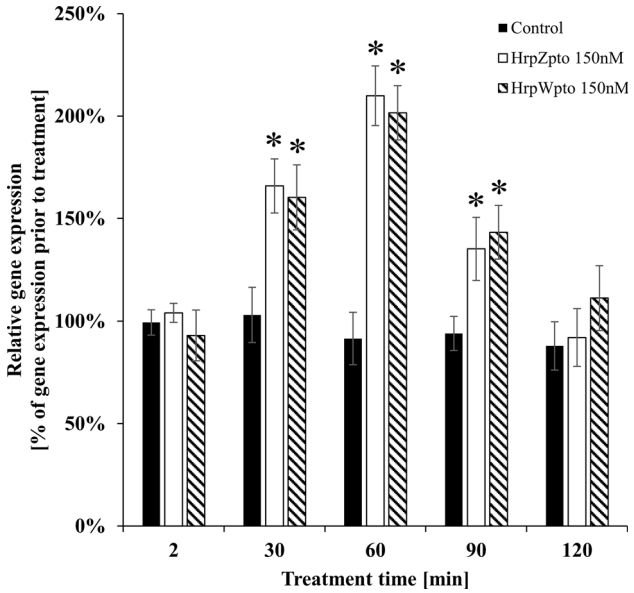

Fig. 3 Relative mRNA level of AOX1a in harpin-treated and nontreated *Arabidopsis* suspension cells. *Arabidopsis* suspension cells were treated with $HrpW_{pto}$ and $HrpZ_{pto}$ at 150 nM final concentration. At indicated time point, samples were taken and total RNA was extracted from them. The quantitative RT-PCR was carried on using specific primers designed from the coding sequences of AOX1a, and mitosis protein YLS8 (as control) genes. For each sample, the transcript levels were normalized with those of mitosis protein YLS8. Data are expressed as means ± SD from three independent harpin treatments. (*Asterisk*) Significant difference with respect to control ($P < 0.01$)

2004; Camejo et al. 2016). It can be observed after the contact of plant cells and pathogen-derived elicitors, such as harpin proteins (Krause and Durner 2004; Reboutier et al. 2007). ROS play dual role in the plant–pathogen interaction. On one hand, they can serve as secondary messengers mediating defence gene activation (Camejo et al. 2016), interact with phosphorylation cascades (Kovtun et al. 2000; Mou et al. 2003), and generate secondary metabolites to arrest pathogen (Thoma et al. 2003); furthermore, they have a role in the induction of HR, that is associated with systemic acquired resistance (Feys and Parker 2000). On the other hand, the overproduction of ROS is definitively toxic to the plant cell itself (Apel and Hirt 2004). Two other observations underline the importance of the fine-tuning of oxidant–antioxidant balance: (1) a threshold exposure time of cells to H_2O_2 was required to initiate irreversibly those processes leading to cell death. This 'presentation time' or exposure time was approx. 60 min (Desikan et al. 1998). (2) The removal of ROS during pathogen or elicitor challenge undoubtedly reduced the rate of HR (Desikan et al. 1998; Levine et al. 1994). Furthermore, the ascorbate biosynthesis-deficient (low ascorbic acid) mutant vtc1-1 and vtc2-1 *A. thaliana* plants both have increased resistance to infection by virulent pathogens (Barth et al. 2004; Pavet et al. 2005).

Since ascorbate is the major water soluble antioxidant in plant cells (Szarka et al. 2012), it must play key role in this oxidant–antioxidant fine-tuning during the early phase of plant–pathogen interaction. On the contrary of this well-established assumption, no full description (biosynthesis and recycling) of the regulation of ascorbate level during elicitor induced oxidative burst in plant cells has been done up to date.

To fill this scientific gap, the main regulatory points of biosynthesis (vtc2, vtc5, and GLDH), recycling (ascorbate–glutathione cycle), and the level of ascorbate was monitored in *A. thaliana* cells due to two different harpin ($HrpZ_{pto}$ and $HrpW_{pto}$) treatments. Since the first 60 min of the treatment are critical (Apel and Hirt 2004; Desikan et al. 1996, 1998), we focused to the events of the first

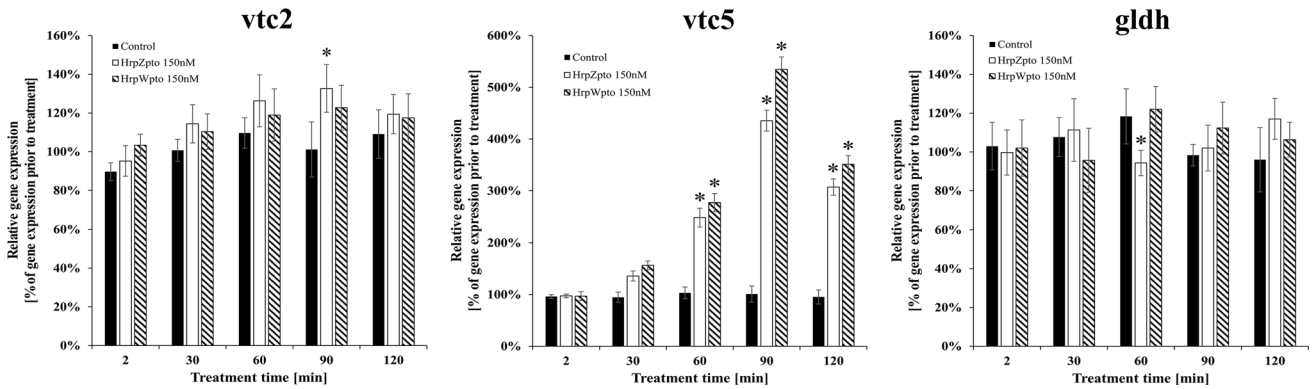

Fig. 4 Relative mRNA level of vtc2, vtc5, and gldh in harpin-treated and nontreated *Arabidopsis* suspension cells. *Arabidopsis* suspension cells were treated with $HrpW_{pto}$ and $HrpZ_{pto}$ at 150 nM final concentration. At indicated time points, samples were taken and total RNA was extracted from them. The quantitative RT-PCR was carried on using specific primers designed from the coding sequences of vtc2, vtc5, gldh, and mitosis protein YLS8 (as control) genes. For each sample, the transcript levels were normalized with those of mitosis protein YLS8. Data are expressed as means ± SD from three independent harpin treatments. (*Asterisk*) Significant difference with respect to control ($P < 0.01$)

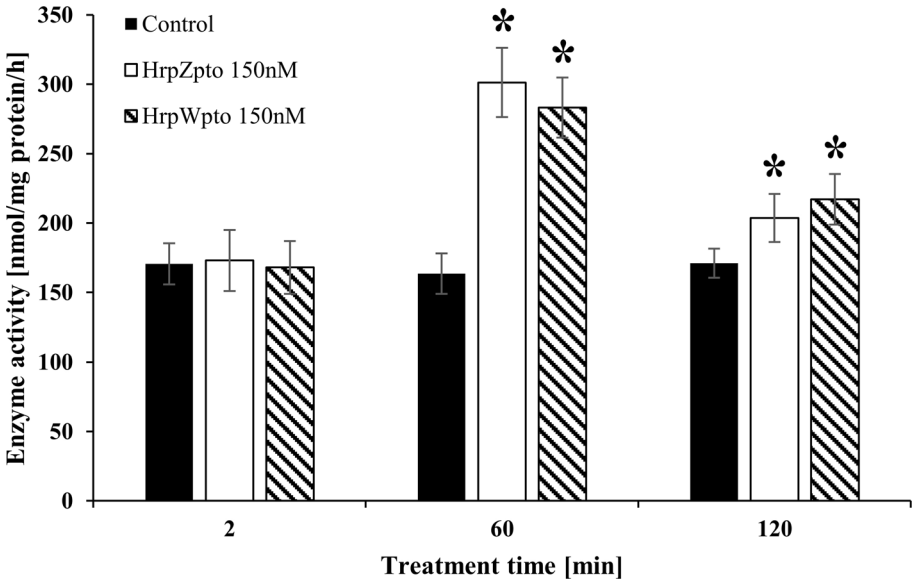

Fig. 5 Effect of harpin treatments on the activity of L-galactono-1,4-lactone dehydrogenase in *Arabidopsis* suspension cells. *Arabidopsis* suspension cells were treated with $HrpW_{pto}$ and $HrpZ_{pto}$ at 150 nM final concentration. At indicated time points, samples were taken. The L-galactono-1,4-lactone dehydrogenase (GLDH) activities were determined from freshly purified mitochondria from harpin-treated and nontreated *A. thaliana* suspension cells as described in "Materials and methods". Data are expressed as means ± SD from three independent harpin treatments. (*Asterisk*) Significant difference with respect to wild type ($P < 0.01$)

120 min. According to the results of Desikan et al. (1996, 1998) and Reboutier et al. (2007), a well-defined ROS peak could be observed 60 min after the $HrpZ_{pto}$ and $HrpW_{pto}$ treatments (Fig. 2, panel a, b). The level of ROS was fallen down with similar pattern in all cases (Fig. 2, Desikan et al. 1996; Reboutier et al. 2007). Enhanced cell death could not be observed in our experiments (Fig. 1). Contrarily, Desikan et al. (1996, 1998) and Reboutier et al. (2007) find a moderate (16–20%) decrease in the cell viability due to harpin treatments. Although the later study used higher (200 nM) harpin concentration (Reboutier et al. 2007) that could cause, the slight difference observed in the cell viability.

The mitochondrial electron transfer chain in plant cells includes an alternative oxidase (AOX) that directly couples the oxidation of ubiquinol to the reduction of oxygen to water (Vanlerberghe 2013). It does not have proton pumping activity and its activity bypasses the proton pumping complex III and IV hence decreasing the generation of O_2^- and NO. Not surprisingly, the induction of AOX1a was described in harpin-induced oxidative burst more than a decade ago (Krause and Durner 2004). Indeed, the enhanced generation of ROS was well followed by the increase of the mRNA level of AOX1a in our experiments (Figs. 2, 3). Although AOX expression responds strongly to bacterial infection (Vidal et al. 2007; Lacomme and Roby 1999; Simons 1999; Amirsadeghi et al. 2007; Sun et al. 2012), it is hard to clearly define the relationship between the changes of AOX amount and activity, the type of bacterial interaction (compatible or incompatible), and downstream responses, such as the HR or changes in defence gene expression (Vanlerberghe 2013). In the experiments of Cvetkovska and Vanlerberghe (2012), the inoculation of *Nicotiana tabacum* with *Pseudomonas syringae* incompatible pv. *maculicola* resulted in a rapid and sustained burst of superoxide, no change in the amount of AOX, and significant rate of HR. However, inoculation with incompatible pv. *phaseolicola* resulted in elevated AOX, no O_2^- burst, and no HR. Later, an O_2^- burst could be generated in AOX knockdown plants due to pv. *phaseolicola* infection by the same group (Cvetkovska and Vanlerberghe 2013). The same AOX knockdown plants infected with pv. *maculicola* showed a delayed O_2^- burst that resulted in a delayed HR. In the light of these results, the lack of cell death (HR) in our case may be explained by the induction of AOX1a (Fig. 3). It cannot be ruled out that the harpin-induced cell death (HR) described by Reboutier et al. (2007) and Desikan et al. (1998) at least partially evolved in the lack of AOX induction. Unfortunately, the AOX expression was not investigated in these studies.

In the next turn of our experiments, the main regulatory points of ascorbate biosynthesis, the expression of vtc2, vtc5, and gldh was investigated. The expression of vtc2 was not affected by harpin treatments practically. Only the $HrpZ_{pto}$ treatment caused a slight, barely significant elevation of vtc2 at 90 min (Fig. 4, panel a). However, the expression of vtc5 was strongly enhanced by both harpin proteins (Fig. 4, panel b). The induction of vtc5 is strongly

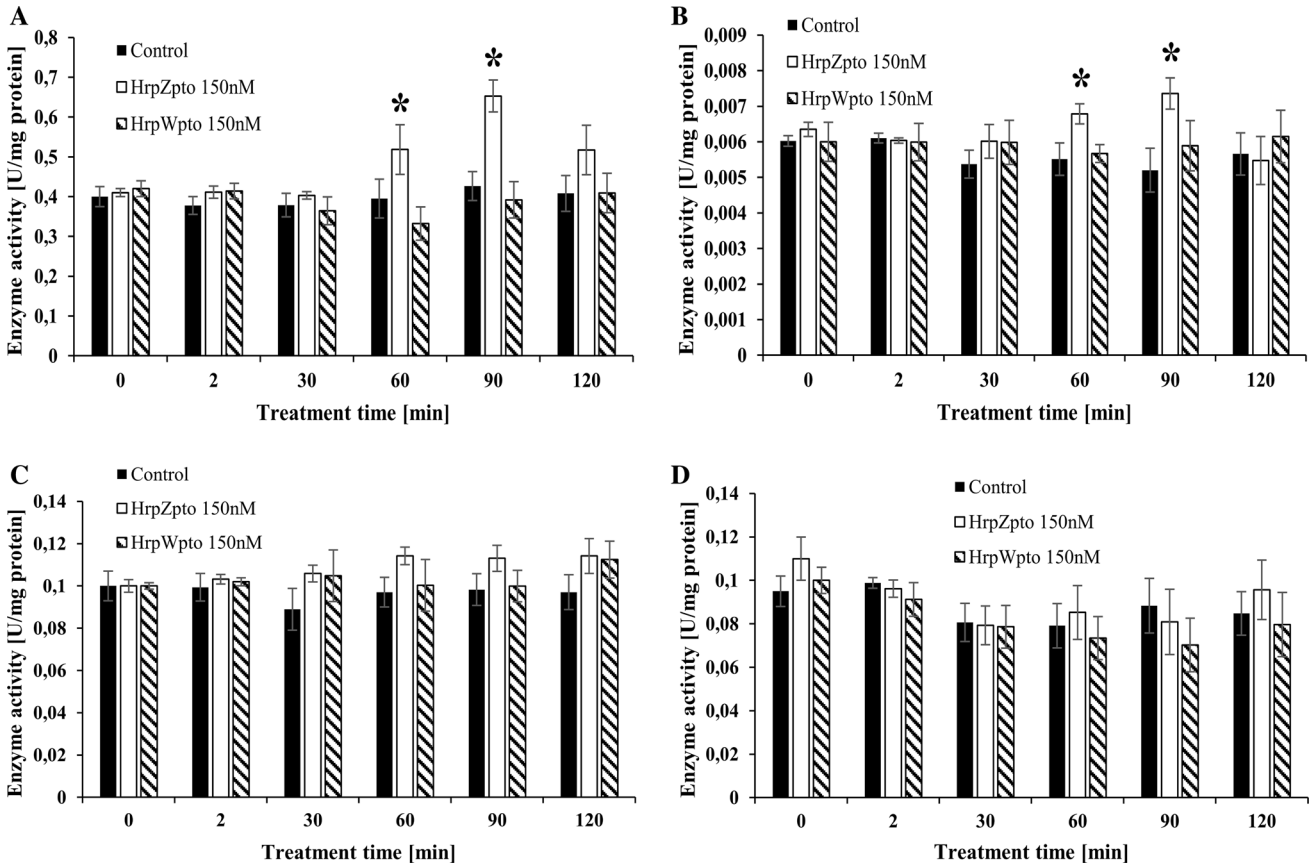

Fig. 6 Activity of the enzymes of the ascorbate–glutathione cycle in harpin-treated and nontreated *Arabidopsis* suspension cells. *Arabidopsis* suspension cells were treated with $HrpW_{pto}$ and $HrpZ_{pto}$ at 150 nM final concentration. At indicated time points, samples were taken, and ascorbate peroxidase (**a**), monodehydroascorbate reductase (**b**), dehydroascorbate reductase (**c**), and glutathione reductase (**d**) activities were determined as described in "Materials and methods". Data are expressed as mean ± SD from three independent harpin treatments. (*Asterisk*) Significant difference with respect to wild type ($P < 0.05$)

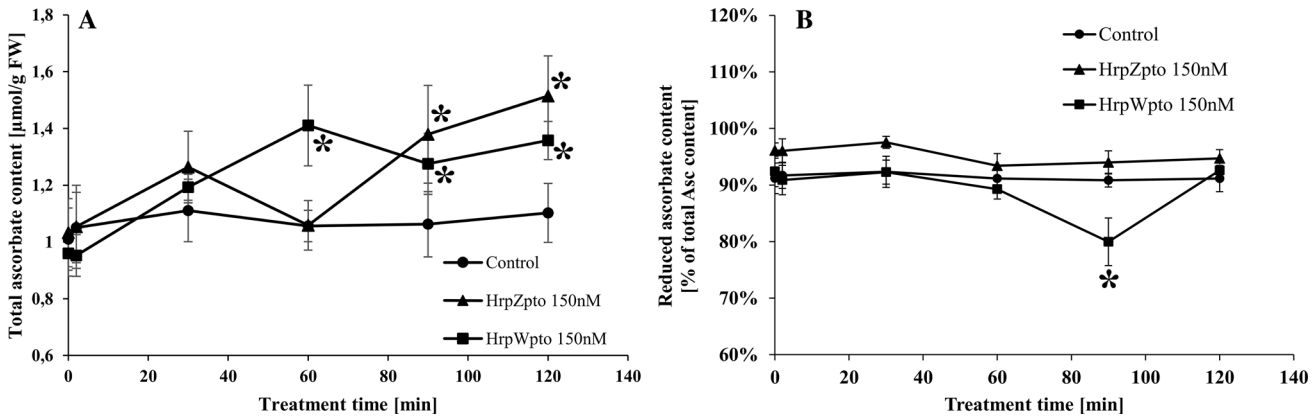

Fig. 7 Effect of harpin treatments on the total ascorbate (**a**) content and redox status (**b**) of *Arabidopsis* suspension cells. *Arabidopsis* suspension cells were treated with $HrpW_{pto}$ and $HrpZ_{pto}$ at 150 nM final concentration. At indicated time points, samples were taken and the ascorbate and dehydroascorbate content of each sample was determined by HPLC as described in "Materials and methods". Data are expressed means ± SD from three independent harpin treatments. (*Asterisk*) Significant difference with respect to control ($P < 0.05$)

significant in the whole investigated period (except at 2 min) (Fig. 4, panel b). It reached its peak at 90 min with a value of 435–535%. The two genes (vtc2 and vtc5) are paralogs and encode the GDP-L-Gal phosphorylase. The enzyme catalyses the committed and mostly regulated step of the major ascorbic acid biosynthetic (Smirnoff-Wheeler) pathway. Both genes are expressed in almost all *Arabidopsis* tissues and regulated by diurnal rhythm and light

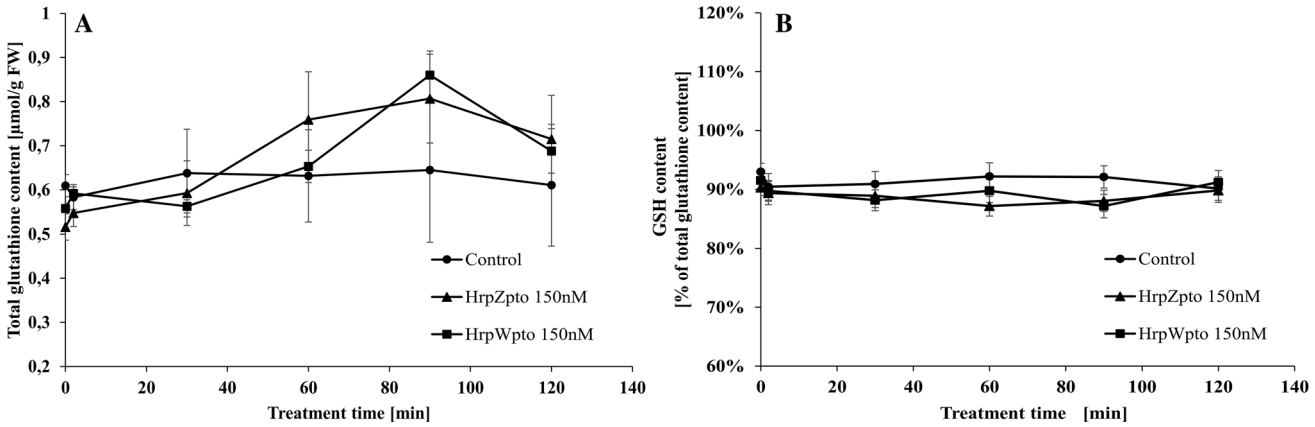

Fig. 8 Effect of harpin treatments on the glutathione (**a**) content and redox status (**b**) of *Arabidopsis* suspension cells. *Arabidopsis* suspension cells were treated with $HrpW_{pto}$ and $HrpZ_{pto}$ at 150 nM final concentration. At indicated time points, samples were taken and the GSH and GSSG content of each sample was determined by HPLC as described in "Materials and methods". Data are expressed means ± SD from three independent harpin treatments

intensity (Dowdle et al. 2007). The expression level of vtc2 in mature leaves is generally 100 to 1000-fold higher than that of vtc5. The expression of vtc2 exceeded that of vtc5 in our nontreated *Arabidopsis* suspension cells too, though its rate was only 4.27-fold higher. Although both vtc2-1 and vtc5-1 mutants lack vtc2 or vtc5 transcripts, vtc2-1 mutant can be characterized by only 20% ascorbate content of the wild type, while vtc5 have about 80% ascorbate content of the wild type (Conklin et al. 2000; Dowdle et al. 2007). Therefore, it is likely that vtc2 has a dominant function in ascorbate biosynthesis, while vtc5 has a limited role. Our results suggest that the paralogs are regulated differentially, at least in harpin-induced oxidative burst. The role of vtc5 might also be the fine-tuning of the redox conditions in the plant–pathogen interaction. Necessarily, it cannot be ruled out that it is a more general role for vtc5. This assumption is strengthened by the observation of Gao et al. (2011) who find that the expression of vtc5 compensated for the reduced vtc2 transcription levels in the ascorbate-deficient mutant vtc2-1 in young *Arabidopsis* seedlings.

The rate limiting role of GLDH in ascorbate biosynthesis is not likely, since tobacco plants overexpressing gldh did not have elevated ascorbate content (Imai et al. 2009), or silencing of GLDH in tomato did not reduce ascorbate levels or its redox status, despite decreased mRNA, protein levels, and enzyme activity (Alhagdow et al. 2007). However, the expression of gldh in rice—similar to the vtc2, vtc5 couple—exhibited light regulation, and the promoter region of gldh contained a conserved light-responsive cis-element-like GT1 box and a TGACG motif (Fukunaga et al. 2010). Furthermore, the rate of ascorbate synthesis is affected by the electron flow through mitochondrial complex I (Millar et al. 2003) and this must be accelerated in harpin-induced oxidative burst (Krause and Durner 2004; Garmier et al. 2007; Cvetkovska and Vanlerberghe 2013). Thus, the effect of harpin treatment on the expression and activity of the sole mitochondrial enzyme of the Smirnoff-Wheeler pathway, GLDH was also investigated. As it could be expected, the expression of gldh did not change due to harpin treatment (Fig. 4, panel c), but its activity followed the level of ROS (rate of oxidative burst) (Fig. 5). This phenomenon is not surprising, since it has been shown that GLDH is an integral protein of the inner mitochondrial membrane (Bartoli et al. 2000) and that cytochrome c_{ox} is the electron acceptor for the GLDH reaction. As a consequence of the absolute requirement of the enzyme for oxidized cytochrome c as a substrate, ascorbate production could be stimulated by anything that reduces the electron flow through the cytochrome c pathway. The described increased expression and activity of AOX (Cvetkovska and Vanlerberghe 2013; Fig. 3) in turn resulted in reduced electron flow through the cytochrome pathway that can result in more oxidized cytochrome c pool. The elevated activity of the mitochondrial ascorbate biosynthesizing enzyme can easily be explained by these events.

The level of ascorbate is determined by its biosynthesis, recycling (from the oxidized form DHA), and by the ascorbate consuming reactions. Oxidized ascorbate can be regenerated at the expense of glutathione or NADPH by the enzymes of the ascorbate–glutathione cycle (Szarka 2013). Thus, the effect of harpin treatment on the activity of all four enzymes of the cycle was also investigated. The $HrpW_{pto}$ treatment had almost no effect on any enzyme of the cycle (Fig. 6). Although the activity of both the APX and the MDHAR was enhanced significantly by $HrpZ_{pto}$ treatment, the elevation of MDHAR was only moderate. Furthermore, $HrpZ_{pto}$ similar to the $HrpW_{pto}$ treatment did not have any effect on the activity of the remaining

enzymes (DHAR and GR) (Fig. 6). The time course of the activity of APX and MDHAR followed the time course of ROS (Figs. 2, 6, panel a, b), both enzymes reached their maximal value after 90 min and then both descended. Since APX has no role in the recycling of ascorbate (rather in the elimination of H_2O_2) (Szarka et al. 2012), we can say that harpin treatments had hardly any effect on the ascorbate–glutathione cycle. In accordance with this really moderate response of the ascorbate–glutathione cycle, $HrpZ_{pto}$ caused only a minor and $HrpW_{pto}$ a bit more pronounced redox shift of ascorbate to the direction of DHA (Fig. 7, panel b).

The enhanced activity of ascorbate biosynthesis raises the question: Is it realized in the elevation of ascorbate level? Is this elevation and the harpin treatment together have any effect on the level and redox state of ascorbate?

Both $HrpZ_{pto}$- and $HrpW_{pto}$-treated cells showed increased (35–50%) total ascorbate contents (Fig. 7, panel a). However, the total ascorbate content of $HrpW_{pto}$-treated plant cells showed a 30 min delay in time compared to $HrpZ_{pto}$ treated with a local maximum value at 60 min (Fig. 7, panel a). This so rapid response in ascorbate level (it elevated within 30–60 min) cannot be explained by the elevation of the mRNA level of vtc5 (Fig. 4, panel b). The observed elevation of GLDH activity (Fig. 5), however, can easily explain the rapid elevation of ascorbate content. Since the activity of GLDH and the synthesis of ascorbate are partly controlled by the respiratory electron flux (Millar et al. 2003; Szarka et al. 2013; Cvetkovska and Vanlerberghe 2013), it can respond quickly to the altered redox conditions and mitochondrial electron flow caused by the oxidative burst.

Similar to the ascorbate content, an elevated level of glutathione could also be observed in the harpin-treated plant cells (Fig. 8). Since the activity of DHAR did not elevated (Fig. 6, panel c), the glutathione requirement of ascorbate–glutathione cycle cannot account for the elevated level of glutathione. It may have a role in the elimination of excess H_2O_2 by the activity of GPXs (Levine et al. 1994; Szarka et al. 2012).

In general, the earlier assessment can be strengthened that ROS seems to orchestrate at least a part of the responses to plant–pathogen interaction. Based on the results, the following model can be hypothesized: The expression of AOX1a (Fig. 2), vtc5 (Fig. 4, panel b), the activity of APX (Fig. 6, panel a), and GLDH (Fig. 5), and, finally, the level of ascorbate (Fig. 7, panel a) and glutathione (Fig. 8) all follow the elevation of ROS due to harpin ($HrpZ_{pto}$, $HrpW_{pto}$) treatment. The enhanced expression of AOX1a (Fig. 2), the elevated activity of GLDH (Fig. 5), and level of ascorbate (Fig. 7, panel a) and glutathione (Fig. 8) all can contribute to the mitigation or absence of programmed cell death (HR) (Fig. 1, Cvetkovska and Vanlerberghe 2012). At the same time, we can propose a new function—beyond the compensation of vtc2 depletion (Gao et al. 2011)—to vtc5, the fine-tuning of redox balance during plant–pathogen interaction. Since the regulation of vtc5 is a black box at this moment, further work is needed to verify this assumption.

Author contribution statement Ádám Czobor and Péter Hajdinák carried out the experiments, collected, and organized data. András Szarka, corresponding author, raised the hypothesis, designed and supervised the experiments, and wrote the manuscript.

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