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Research article

Dormancy removal by cold stratification increases glutathione and *S*-nitrosoglutathione content in apple seeds

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ABSTRACT

S-nitrosoglutathione (GSNO), an integral metabolite of nitric oxide (NO) biochemistry is reduced by *S*-nitrosoglutathione reductase (GSNOR) (EC 1.2.1.46), leading to formation of glutathione in oxidised form (GSSG), further reduced to GSH by glutathione reductase (GR). GSH as a vital antioxidant has a significant role for seed quality and during seed germination. Since early 50th of 20th century it is known that deep dormancy of apple (*Malus domestica* Borkh.) embryos is removed by 90 days of cold stratification. Our previous studies demonstrated that similar effect is observed after short term (3 h) exposition of isolated embryos to nitric oxide (NO) donors. The aim of our work was to verify the differences in GSNO level and GSNOR activity in embryonic axes isolated after initiation of germination (24 h of imbibition) from dormant embryos (the control) and from 90 days cold stratified seeds. Our data indicated that seed dormancy breakage is accompanied by increased GSNO content and the decrease of GSNOR activity. The abundance of GSNOR protein is similar in both non-dormant and dormant embryonic axes during first hours of water uptake, while *GSNOR* transcript level increases in non-dormant tissue. Furthermore, in non-dormant embryonic axes we noticed a higher glutathione pool, mostly in its reduced form. These results are linked to the increase of cytosolic GR transcript level and increased enzyme activity in embryonic axes isolated from stratified seeds.

1. Introduction

The establishment of a dormancy state was an evolutionary strategy, permitting plants to avoid unfavourable environmental conditions. Seed dormancy is commonly defined as a temporal arrest of growth and development of viable organs (Hilhorst, 2007). The strength of this physiological state is under control of different endogenous and exogenous factors (Finch-Savage and Leubner-Metzger, 2006; Krasuska et al., 2015a, 2015b). Dormancy release "opens the window" for metabolic events which initiate seed germination (Finch-Savage and Leubner-Metzger, 2006). Seed germination *sensu stricto* is described as the termination of embryo activation with visible effect of an embryonic root protrusion throughout seed covering layers (Finch-Savage and Leubner-Metzger, 2006; Lewak, 2011). Seeds imbibition (at the initial phase of germination) is accompanied by enhanced production of reactive oxygen species (ROS) (Bailly et al., 2008) and reactive nitrogen species (RNS), which is observed also in germinating apple embryos (*Malus domestica* Borkh.) (Gniazdowska et al., 2010a, 2010b). This is linked to a stimulation of cellular antioxidant system (Krasuska and Gniazdowska, 2012).

Seeds of apple belong to the orthodox type characterised by a deep physical and physiological dormancy (Lewak, 2011). Embryos isolated from dormant seeds (after removal of seed coat and endosperm) germinate very slowly (even under favourable conditions) and develop abnormal seedlings with shortened embryonic axes/roots and asynchronously greening cotyledons. Dormancy of apple seeds can be removed by 90 days long cold stratification (Dębska et al., 2013; Lewak, 2011) or after short term (3–6 h) pre-treatment of isolated embryos with donors of nitric oxide (NO) or hydrogen cyanide (HCN) (Bogatek and Gniazdowska, 2006; Gniazdowska et al., 2010b). Cold stratification is a commonly used practice consisting of imbibition of dormant seeds at chilling temperature (above 0 °C) for the period that varies,

Abbreviations: CTCF, corrected total cell fluorescence; $E_{GSSG/2GSH}$, glutathione half-cell reduction potential; GR, glutathione reductase; GSH, reduced form of glutathione; GSNO, *S*-nitrosoglutathione; GSNOR, *S*-nitrosoglutathione reductase; GSSG, oxidised form of glutathione; NO, nitric oxide; RNS, reactive nitrogen species; ROS, reactive oxygen species

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depending on the plant species. Non-dormant apple embryos (stratified/treated with NO or HCN) germinate fast (Gniazdowska et al., 2007, 2010a; 2010b) with chloroplasts developing in both cotyledons and of an equal size (Krasuska et al., 2015b). Elevated HCN (Lewak, 2011 and references herein), ROS and RNS generation is observed in apple seeds (Dębska et al., 2013) during cold stratification. While, application of NO scavengers keeps embryos at the dormancy state (Gniazdowska et al., 2007; Krasuska et al., 2016).

Increasing number of experimental data reveal that both ROS and RNS are multifunctional molecules playing a substantial role in plant physiology and particularly in seed biology (Bailly et al., 2008; Šírová et al., 2011; Yu et al., 2014). Dual function of RNS can be described by the model of "nitrosative door" (similar to "oxidative window" designed by Bailly et al. (2008) for ROS) which "opens" for seed germination when intracellular NO level reaches optimal concentration (Krasuska et al., 2015a). The mode of action of RNS depends on modification of amino acid residues in proteins (posttranslational modification) e.g. nitration or *S*-nitrosation (Tichá et al., 2016; Yu et al., 2014). *S*-nitrosation of proteins or peptides occurs when nitroso group is attached to a cysteine residue. This posttranslational modification has a strong impact on protein structure and/or function (Tichá et al., 2016).

In plant cells, NO is synthesized via oxidative or reductive pathways (Gupta et al., 2011). During seed dormancy alleviation, increased NO level may come from enzymatic or non-enzymatic sources. Till today there is no strong molecular evidence for the presence of NO synthase (NOS) in higher plants (Jeandroz et al., 2016), however arginine (Arg)-dependent NOS-like activity has been detected in several higher plants (Corpas and Barroso, 2017), also in axes of germinating apple embryos (Krasuska et al., 2016, 2017b). It is suggested that non-enzymatic sources of NO are more relevant in seed dormancy breakage, especially during initial hours of imbibition. One of the important NO donor is nitrite (NO_2^-), which liberates NO under acidic pH (Yamasaki, 2000) or acts as acceptor of electrons (Igamberdiev et al., 2010). Alteration in NO_2^- concentration is linked to dormancy loss and stimulation of germination of apple embryos (Krasuska et al., 2017b).

S-nitrosoglutathione (GSNO) is considered as a cellular NO reservoir (Broniowska et al., 2013; Dürner and Klessig, 1999). This molecule is the most abundant *S*-nitrosated derivative thiol of many physiological function (Broniowska et al., 2013). GSNO was detected in various plant tissues (Barroso et al., 2006) and quite recently also in seeds (Ma et al., 2016). This molecule is more stable than *S*-nitrosocysteine (Bartberger et al., 2000, 2001) and in specific conditions (presence of transition metal ions e.g. copper) can liberate NO (review by Broniowska et al., 2013). Transnitrosation is the physiologically relevant mechanism of GSNO decay when the nitroso functional group is donated to another thiol (Hogg, 1999).

GSNO reductase (GSNOR) (EC 1.2.1.46) is a glutathione-dependent enzyme, a member of class III alcohol dehydrogenase family (Jensen et al., 1998; Tichá et al., 2016). In plants growing under optimal conditions activity of GSNOR is linked to physiological development and fertility (Lee et al., 2008). In Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.) it was demonstrated that this enzyme was not expressed at the same level in all parts of the seedling and the activity varied depending on organ and developmental stage (Espunya et al., 2006). Most data describing GSNOR *in planta* are focused on its role in reaction to abiotic and biotic stresses (Barroso et al., 2006; Kubienová et al., 2014). Reduction of GSNO by GSNOR leads to formation of oxidised form of glutathione (GSSG) and NH_2OH or alternatively ends with the rearrangement and then spontaneous hydrolyzation to GSO_2H and NH_3 (Jensen et al., 1998). GSNOR activity is not connected with NO liberation, rather with effective scavenging from the free "NO pool", thus this enzyme regulates nitrosylated glutathione level (Broniowska et al., 2013; Ma et al., 2016). Activity of this enzyme has been confirmed in many plants (Airaki et al., 2012; Barroso et al., 2006; Chaki et al., 2011; Krasuska et al., 2017a; Kubienová et al., 2013, 2014). In Arabidopsis GSNOR is encoded by one gene, not expressed only in mature pollen (Leterrier et al., 2011).

Glutathione in reduced form (GSH, the tripeptide, γ-glutamyl cysteinyl glycine) is a non-enzymatic low-molecular-weight antioxidant necessary for the maintenance of a cellular redox state (Noctor et al., 2012). At the stage of the transition from germination to seedling growth the process of cell division depends on increasing GSH content (Diaz-Vivancos et al., 2010). In orthodox seeds at the phase of early germination, this tripeptide is the most important antioxidant (Tommasi et al., 2001). Contrary, overaccumulation of GSSG is toxic and results in loss of viability. Glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) correlates with cell viability and can be used as a valuable marker to predict seed germination potential (Kranner et al., 2006). Glutathione reductase (GR, EC 1.6.4.2) is NADPH dependent enzyme responsible for reduction of GSSG. Using transgenic Arabidopsis lines overexpressing *GSNOR* or with antisense *GSNOR* it was confirmed that redox homeostasis is also under GSNOR regulation. Roots of plants of tested lines were characterised by a lower intracellular GSH level than observed in wild type (Espunya et al., 2006).

Our previous results demonstrated a pivotal role of RNS in release of apple embryo dormancy (Bogatek and Gniazdowska, 2006; Gniazdowska et al., 2007, 2010a, 2010b). Nevertheless, the main intracellular source and regulation of availability of these molecules at the beginning of germination *sensu stricto* remain unclear and need further investigation. The aim of this work was to demonstrate experimental evidence for GSNO occurrence and variation in its level depending on dormancy status of the embryos. Our study was focused on determination of GSNOR protein level, catalytic activity and gene transcription in axes of dormant and non-dormant embryos after initial 24 h of water uptake (imbibition). We linked GSNO level and GSNOR activity to the total glutathione pool as well as GSH/GSSG ratio. Previous data indicated that apple embryos dormancy release stimulated by NO fumigation was accompanied by increased GR activity, preventing GSSG overaccumulation (Krasuska and Gniazdowska, 2012). Therefore, in the current research we studied GR activity and transcription in axes of non-dormant embryos isolated from stratified seeds.

2. Material and methods

2.1. Plant material

Apple (*Malus domestica* Borkh., cv. Antonówka) seeds were isolated from fruits, dried and stored in a glass container at 5 °C (dormant seeds). To overcome dormancy, seeds were placed in glass Petri dishes filled with sterile sand moistened with distilled water for 90 days at 5 °C. After cold stratification seeds were removed from sand, washed in distilled water, dried to moisture content below 10% and stored in a glass container at 5 °C until use.

Dormant and non-dormant seeds were imbibed in distilled water for 24 h at room temperature. After imbibition seed coat and endosperm were removed and embryos were washed in distilled water. Isolated embryonic axes were taken for further analyses.

Germination of the embryos was checked after 24 h. Experiment was repeated 5 times, using 20 embryos per each.

2.2. Detection of GSNOR protein

2.2.1. Protein isolation

Axes of apple embryos (approx. 30 mg) were homogenized in 0.1 M Tris-HCl (pH 8.0) buffer containing 1 mM EDTA, 2% (w/v) polyvinylpolypyrrolidone (PVPP), 5 mM dithiothreitol (DTT), 1 mM $MgCl_2$, 1% (v/v) protease inhibitor cocktail, 10% glycerol, and 0.1% (w/v) Triton X-100 in ice bath. After centrifugation at 12,000 *g* for 10 min at 4 °C supernatant was collected and desalted using concentrator PES, 3K MWCO (Thermo Scientific) at 10,000 *g* for 20 min at 4 °C. Concentration of proteins was measured according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.2.2. SDS-PAGE separation of proteins

Collected proteins extracts were suspended in the buffer containing 63 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 20 mM DTT and incubated at 95 °C for 5 min. 15 μg of total proteins were loaded per line and separated on 10% standard SDS-polyacrylamide gels (SDS-PAGE) according to Laemmli (1970).

2.2.3. Western-blot of proteins and GSNOR detection

After SDS-PAGE separation proteins were electrotransferred onto nitrocellulose membranes (Amersham™ Protran™ 0.2 NC) according to Towbin et al. (1979) using a Bio-Rad wet electroblotting system. After transfer the membranes were stained for protein visualization using 0.2% Ponceau Red in 2% acetic acid solution. Nitrocellulose membranes were blocked overnight at 4 °C with 5% (w/v) nonfat dry milk dissolved in TBST buffer. Immunolabelling was carried out by incubation of the membranes with the primary antibodies (Agrisera, AS09 647) at recommended dilution for 1.5 h. After wash steps with TBST, secondary antibodies conjugated with an alkaline phosphatase (Agrisera, AS10 1458) were used according to the procedure. After 1 h of incubation, the membranes were washed three times with TBST. Visualization of the protein was performed using a mixture of 0.2 mM nitroblue tetrazolium salt (NBT) and 0.21 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in buffer containing 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$. Assays were done in 3–4 independent experiments and their typical results are shown.

2.3. Measurement of GSNOR activity

GSNOR activity was measured as described by Sakamoto et al. (2002) with some modifications. Embryonic axes (approx. 30 mg) isolated from dormant and non-dormant embryos were ground in 0.6 ml of the extraction buffer (50 mM Tris-HCl pH 8.0, 5 mM DTT, 5% (w/v) glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 1% (v/v) protease inhibitor cocktail and 2% (w/v) PVPP) on ice, well mixed and centrifuged at 10,000 *g*, 4 °C for 10 min. Extract was desalted using concentrator PES, 3K MWCO (Thermo Scientific) at 10,000 *g* for 20 min at 4 °C. The reaction mixture consisted of 260 μl of 50 mM Tris-HCl pH 8.0, containing 0.5 mM EDTA and 0.2 mM NADH and 20 μl of protein extract. The enzymatic reaction was started by the addition of 20 μl of 9 mM GSNO. In parallel, the reaction for all samples was also carried out in the absence of GSNO.

GSNOR activity was measured for 6 min, at 340 nm with microplate reader (Sunrise, Tecan). Results were calculated using the molar extinction coefficient for NADH ($\mathcal{E} = 6220\ M^{-1}cm^{-1}$). Protein concentration in extract was measured using Bradford reagent (Bradford, 1976). GSNOR activity was expressed as nmol min^{-1} mg^{-1} protein. Experiments were done in at least three biological repetitions, in three technical replicates each.

2.4. Immunolocalization of GSNO

Axes of apple embryos were immediately fixed in 4% (w/v) paraformaldehyde in 0.1 M microtubule stabilizing buffer (MSB), pH 6.9 with 0.1% (v/v) Triton X-100 for 2 h at room temperature as described by Gubler (1989). Samples were dehydrated in ethanol with 10 mM DTT and infiltrated in a butyl-methyl-methacrylate resin (BMM) with ethanol in dilution: 1:3, 1:1, 3:1, and finally, in pure BMM. Polymerization was performed for 20 h at −20 °C. Acetone was used to remove the BMM from 2.0 μm - sections collected on silane coated-slides (Thermo-Fischer Scientific, Poland). Immunofluorescence analysis was carried out after pre-incubation in 3% (w/v) BSA in phosphate-buffered saline (PBS) containing 3.2 mM Na_2HPO4, 0.5 mM KH_2PO_4, 135 mM NaCl, 1.3 mM KCl, pH 7.2 for 1 h at room temperature. Sections were incubated with primary rat anti-GSNO antibodies (Agrisera, AS08 361) in PBS buffer (pH 7.2) in dilution 1:1000 for 2 h at room temperature in a humid chamber. Controls for staining were performed by replacing the primary antibody with pre-immune serum. Slides washed with PBS-Tween 20 buffer were treated at room temperature in the darkness for 1.5 h with the secondary goat anti-rat IgG conjugated to TexasRed-X (EX 596, EM 615; Thermo-Fischer Scientific, T-6392) in PBS buffer diluted 1:1000 in a humid chamber. An Olympus AX70 Provis (Olympus Poland, Warsaw, Poland) with a UM61002 filter set and equipped with an Olympus SC35 camera was used for fluorescence imaging.

Quantitative measurement of the fluorescence signal were performed according to Burgess et al. (2010). As a first step of analyses, cells with the red fluoresce signal from GSNO epitope were marked and assessed by use of ImageJ program. Fluorescence signal was calculated in a form of corrected total cell fluorescence (CTCF) using the formula:

CTCF = integrated density from labelled root - (area of selected root cells region x mean fluorescence of background readings).

Assays were done in 3–4 independent experiments and their typical results are shown.

2.5. Measurement of GSH and GSSG concentration

GSH and GSSG content was determined using monobromobimane (MBBr; Sigma 69898) as a derivatizing agent. The level of GSH derivatives were measured by reversed phase HPLC method with fluorescence detection according to Queval and Noctor (2007) with some modification.

Embryonic axes (approx. 30 mg) isolated from dormant and non-dormant embryos were ground in liquid nitrogen and homogenized in 0.4 ml of 50 mM HCl with 2% (w/v) PVPP. After centrifugation (12,000 *g*, 15 min, 4 °C), 200 μl of extract was mixed with 12.5 mM HEPES-KOH to adjust pH of the solution to 7.5. The derivatization was done by adding 15 μl of 20 mM MBBr (dissolved in methanol) to 100 μl of extract. After 15 min of incubation at room temperature in the darkness, samples were centrifuged and 10 μl of solution was injected into HPLC system. To determine GSSG content, after pH adjustment, GSSG (100 μl of extract) was reduced with 1.5 μl of 100 mM DTT in the darkness at room temperature for 30 min, which was followed by derivatization described above.

Separation of bromine derivatives was achieved using Bionacom Velocity C18 LPH (4.6 · 150; 3 μm) column, kept at thermostat with stable temperature of 35 °C. Peak was detected using the FP-2020/2025 Intelligent Fluorescence Detector (JASCO) (E_x 390 nm; E_m 478 nm). Acetic acid 0.25% (A) and methanol 100% (B) were used as a mobile phase. The flow rate of eluents was 1 ml min^{-1}. Compounds were eluted by the following gradient: 0–5 min 80-75% (A), 5–30 min 75-70% (A), 30–38 min 70-0% (A), 38–45 min 0–80% (A).

Standard curve of GSH was prepared in 50 mM HCl, derivatized with MBBr and analyzed as described above. Measurements were done in four biological replicates, each in three technical replicates. GSH and GSSG concentration was presented as nmol g^{-1} FW.

2.5.1. Glutathione half-cell reduction potential

$E_{GSSG/2GSH}$ (in mV) was calculated based on the Nernst equation (1) (Kranner et al., 2006; Morscher et al., 2015).

$$E_{GSSG/2GSH} = -E^{0'} - RT\,[nF]^{-1} \ln [GSH^2\ GSSG^{-1}] \quad (1)$$

where R, gas constant (8.314 $JK^{-1}mol^{-1}$); T, temperature in K during storage and stratification (4 °C); n, number of transferred electrons ($2GSH \rightarrow GSSG + 2H^+ + 2e^-$); F, Faraday constant 9.6485 10^4 eV mol^{-1}; E0′, standard half-cell reduction potential of glutathione (−0.240 V) at pH 7.

2.6. Measurement of GR activity

Measurement of GR activity was done according to Esterbauer and Grill (1978) with some modifications. Axes of apple embryos (approx.

25 mg) were ground in 0.6 ml of 50 mM K-phosphate buffer pH 7.5 containing 5 mM DTT, 5% (w/v) glycerol, 1 mM EDTA, 0.1% (w/v) Triton X-100, 1% (v/v) protease inhibitor cocktail and 2% (w/v) PVPP) on ice, well mixed and centrifuged at 10,000 *g*, 4 °C for 10 min. Extract was desalted using centrifugal concentrator PES, 3K MWCO (Thermo Scientific) at 10,000 *g*, 4 °C for 20 min. The reaction mixture consisting of 165 µl of K-phosphate buffer pH 7.5, 25 µl 5 mM GSSG and 35 µl of protein extract was incubated for 10 min at 30 °C. The enzymatic reaction was started by the addition of 25 µl of 2 mM NADPH and was carried out at 30 °C for 6 min. In parallel, the reaction for all samples was also performed in the absence of GSSG.

GR activity was determined as absorbance decrease, monitored at 340 nm using microplate reader (Sunrise, Tecan). Protein concentration in extracts was measured using Bradford reagent (Bradford, 1976). GR activity was expressed as nmol min^{-1} mg^{-1} protein. Experiments were done in 3–4 biological replicates, in three technical repetitions.

2.7. Gene expression analysis

The expression of genes was achieved in axes of apple embryos using qPCR. Total RNA was isolated using RNAzol® RT (Sigma, R4533), according to the manufacturer's guideline. 200 ng of total RNA was taken for cDNA synthesis. The cDNA was generated using RevertAid First Strand cDNA Synthesis Kit (Termo Scientific, #K1622) in a total volume of 10 µl. The finished product was diluted 3.5 times. qPCR was carried out in a Bio Rad CFX Connect™ Real-Time PCR Detection System. iTaq™ universal SYBR® Green supermix (Bio Rad, #172–5124) was used for the reaction in a total volume of 12 µl (6 µl PCR supermix, 1 µl cDNA, 1 µl primer, 4 µl H_2O). Specific primers were designed based on nucleotide sequences available in the GenBank database on the National Center of Biotechnological Information (NCBI) and in the Genome Database for Rosaceae (https://www.rosaceae.org/) (*GRc* F: 5′-CTC TGT TAT CAT GTC GAG GAA GAT GC-3′ R: 5′-ATCTGGAGGC ACGGACACCA-3′; *GRp* F:5′- CCAAAGGTTGTGGCTCCTGA-3′ R: 5′-ACATGCTGCATCTGTGCTCT-3′; *GSNOR* F: 5′-TTCACATATTCCGAT CCCCACA-3′ R: 5′-CCGCTTTGCAGGTGATGATTT-3′). Expression levels were normalized using two reference genes encoding (1) ubiquitin-conjugating enzyme (*UCE;* F: 5′-CCTCCTCTCCATTTGCTCCCTTC-3′ and R: 5′-GAGCAGTGGCCTCGTATTTCTGT-3′) or (2) protein phosphatase 2A (*PP2A*; 5′-TGGACCGTATACAGGAGGTTC-3′ and R: 5′-CTGCCCGAA TGTATAGCCAG-3′) and calculated using the $2^{-\Delta Ct}$ method. Transcript level of the genes in dormant tissue was expressed as 1. Experiments were done in three biological replicates, in three technical repetitions.

2.8. Statistics

All data were obtained in at least 3 independent experiments with at least 2 repetition each. Data were analyzed using the Statistica Software. Mean values were compared by *t*-test, SD was also provided to indicate the variations associated with the particular mean values.

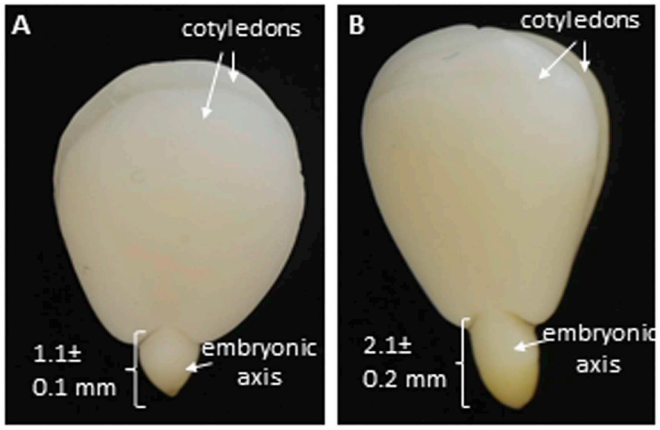
3. Results

3.1. Cold stratification resulted in dormancy release in apple embryos

90 days long cold stratification of apple seeds resulted in dormancy breakage. All embryos isolated from stratified seeds germinated within 24 h (Fig. 1). For all of them elongation of embryonic axes was the first visible effect of dormancy breakage (Fig. 1). In contrast, non of embryos isolated from dormant (control) seeds exhibited elongation of embryonic axes (Fig. 1).

3.2. Dormancy release in apple embryos led to decline of GSNOR activity but did not modify protein content and increased gene transcript level

Content of GSNOR protein in apple axes was investigated by



Embryos	Germination after 24 h (%)
dormant (control)	0
non-dormant (stratified)	100

Fig. 1. Morphology of apple embryos isolated from dormant (control) seed (A) and isolated from non-dormant (stratified) seed (B). Representative embryos are shown. Photos were done using Nikon SMZ 18 Digital Zoom Stereomicroscope. Germination rate of dormant and non-dormant apple embryos after 24 h of imbibition in water. Values are average of 5 independent experiments with 20 embryos in each.

Western blot analysis. Single bands corresponding to protein of molecular weight of about 43 kDa were clearly visible in extracts of axes of dormant and non-dormant embryos (Fig. 2A). In extracts from axes of non-dormant embryos bands corresponding to protein of molecular weight of 43 kDa were similar as in dormant ones.

The GSNOR gene expression in axes of non-dormant embryos was higher than in dormant ones (Fig. 2B and Table A1).

Cold stratification declined GSNOR activity determined in extracts of embryonic axes. It reached around 1 nmol min^{-1} mg^{-1} protein and was twice lower than in axes of dormant embryos (Fig. 2C).

3.3. Dormancy removal was accompanied by enlarged GSNO level and modification of GSNO localization in axes of apple embryos

Localization of GSNO was done on longitudinal sections of axes by single immunofluorescence labelling technique. Microscopic visualization indicated the stronger GSNO deposition in sections of axes after stratification compared to dormant ones. In axes of dormant embryos GSNO red fluorescence signal was detected in external part of the tissue (Fig. 3B). After stratification the red fluorescence signal was mainly observed in cells of root cap and in promeristem divisions zone (Fig. 3C). Microscopic visualization of GSNO deposition in axes, was confirmed by quantification analysis using the corrected total cell fluorescence method (Fig. 3D).

In the tissue with primary antibodies replaced by pre-immune serum, no red fluorescence signal was noticed (data not shown).

3.4. Dormancy alleviation by cold stratification increased total glutathione level in embryonic axes

Total concentration of glutathione (GSH + GSSG) was almost doubled in axes of non-dormant embryos as compared to dormant ones (Table 1). In dormant tissue GSSG reached 12% of total glutathione, while in non-dormant it was 6%. GSH/GSSG ratio were 7.44 for dormant and twice higher for non-dormant embryos (Table 1). Glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) of non-dormant embryonic

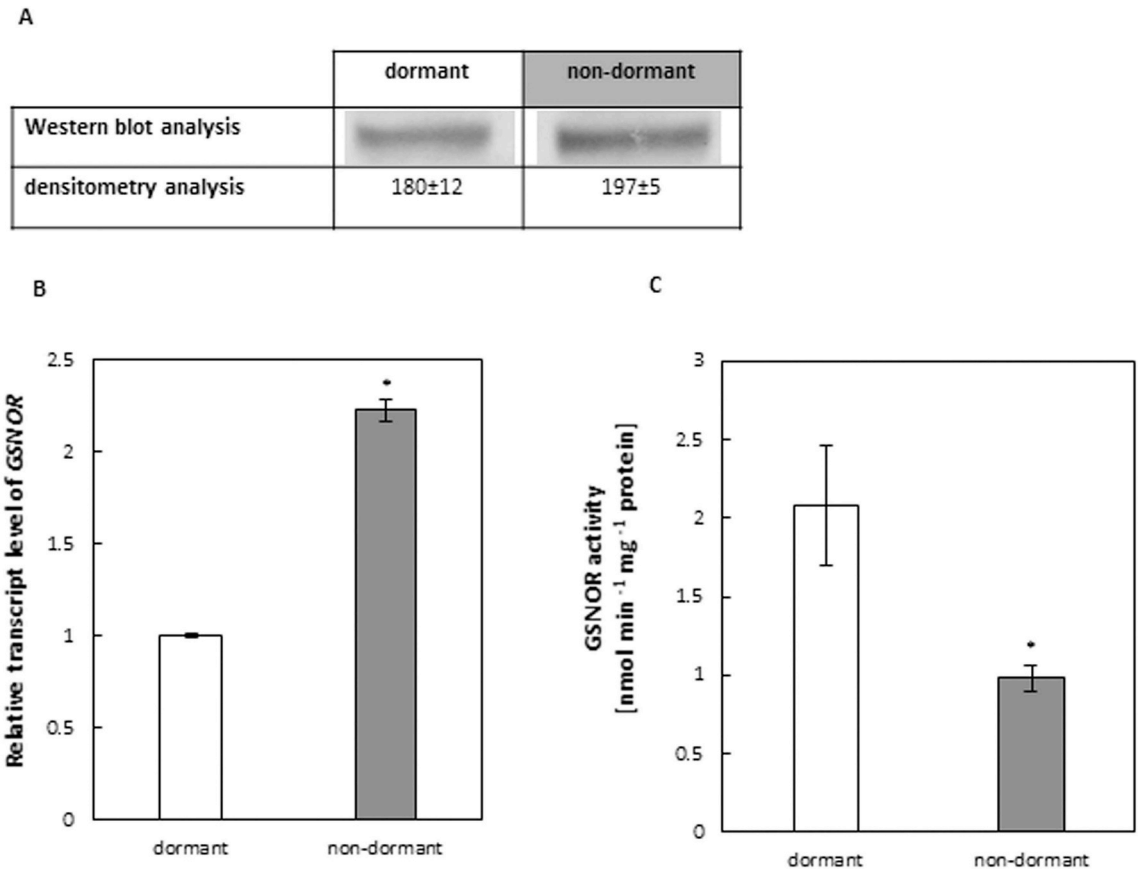


Fig. 2. Amount of GSNOR protein (A), transcript level of *GSNOR* (B) and activity of GSNOR (C) in axes of dormant (control) or non-dormant apple embryos. Expression of *GSNOR* was normalized to *UCE* as a reference gene. Asterisks (*) indicate significance from control at $P \leq 0.05$, based on Student's test. Values are average ± SD of at least 3 independent experiments and 3 biological repetitions each.

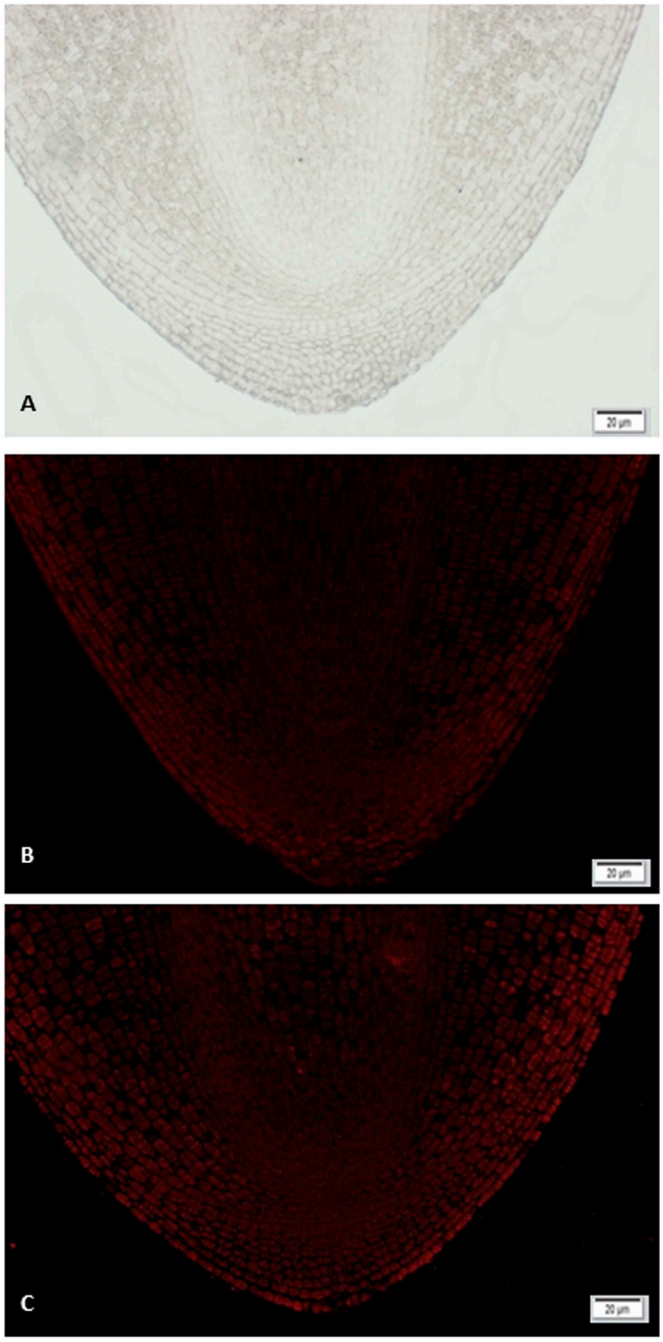
axes was 17 mV more negative that it was in axes of dormant embryos (Table 1). In axes of both dormant and non-dormant embryos $E_{GSSG/2GSH}$ was below −250 mV, indicating strongly reductive conditions.

3.5. Dormancy release in apple embryos stimulated GR activity and transcript level of cytosolic form

Dormancy release by cold stratification resulted in stimulation of GR activity. GR activity in axes of non-dormant embryos was more than doubled as compared to dormant ones (Table 2). It was accompanied by increased transcript levels of gene encoding cytosolic GR. Expression of plastidic form was similar in both axes of dormant and non-dormant embryos (Table 2, Table A1).

4. Discussion

Dormancy is a physiological state when viable seeds do not germinate. Seed stratification, a commonly used technique, is used for dormancy removal and can be performed in moisture for an experimentally revealed time at warm or cold temperatures. All conditions depends on plant species (Dębska et al., 2013; Lewak, 2011). Cold stratification of apple seeds (90 days at 5 °C) remarkably enhances germination rate of isolated embryos (Dębska et al., 2013; Lewak, 2011). These embryos imbibed for 24 h in distilled water are characterised by longer embryonic axes comparing to the control (dormant apple embryos isolated from seeds imbibed for 24 h) (Fig. 1). Based on previously obtained data, dormancy release during cold stratification is accompanied by endogenous increase of NO and ROS (mainly H_2O_2) level (Dębska et al., 2013; Lewak, 2011). Regulation of seed germination by RNS includes a cross-talk with polyamines (Krasuska et al., 2013, 2017b) and depends on protein modification e.g. nitration, carbonylation and/or *S*-nitrosation (Krasuska et al., 2014, 2016; Sen, 2010). In contrast, the maintenance of seed dormancy can be achieved by application of NO scavengers (Gniazdowska et al., 2007). Naturally occurring NO scavengers are class 1 plant hemoglobin (Igamberdiev et al., 2006) and GSNOR as both lower free "NO pool" (Ma et al., 2016). Thus we assumed that activity of GSNOR regulates the strength of apple embryos dormancy. We indicated that GSNOR protein level was similar in axes of dormant and non-dormant apple embryos (Fig. 2A), though its activity was twice lower in non-dormant embryos comparing to the control (Fig. 2C). Lowering activity of this enzymatic NO scavenger goes together with transient increase of RNS level which occurs during embryo dormancy release (Dębska et al., 2013; Gniazdowska et al., 2010c; Krasuska et al., 2013). Opposite, during first hours post imbibition of non-dormant barley (*Hordeum vulgare* L.) embryos besides high NO level, stimulation of GSNOR activity was noticed (Ma et al., 2016). Thus it is the most probably that GSNOR activity depends on plant species, developmental stage and physiological conditions. Ma et al. (2016) demonstrated relatively constant expression of *GSNOR* and they assumed that alteration in enzyme activity could be attributed to the posttranscriptional regulation. In embryonic axes of non-dormant (stratified) apple seeds we observed higher *GSNOR* transcript level (Fig. 2B). Taking together our results concerning GSNOR protein and transcript level as well as enzyme activity we think that besides posttranscriptional there is also posttranslational regulation. As demonstrated by Xu et al. (2013) GSNOR from Arabidopsis has most of the ex-zinc cysteines inaccessible to solvent but three of these amino acids that are positionally conserved are solvent accessible. These residues could serve as sites of posttranslational regulation by glutathionylation or *S*-nitrosation (Xu et al., 2013). Increased NO level in apple embryos during dormancy release



D

Axes of embryos	CTCF value in the tissue
dormant (control) (B)	9283
non-dormant (C)	173937

Fig. 3. Tip of axes of apple embryo, longitudinal section in bright field (A), GSNO localization in axes of embryos isolated from dormant seeds (B), GSNO localization in axes of embryos isolated from non-dormant (stratified seeds) (C), CTCF value in the tissue (D). For GSNO localization representative data are shown. For CTCF values are average ± SD of 4–5 repetitions. Asterisks (*) indicate significance from the control at $P \leq 0.05$, based on student test. Scale bars 20 μm.

possibly could be engaged in GSNOR activity regulation (a negative feedback loop) including *S*-nitrosation. Contrary to the nitration, this modification is reversed, thus if necessary the activation of GSNOR might occur. Just recently other possibility of GSNOR activity regulation was described as induction of selective autophagy by *S*-nitrosation at Cys-10 in Arabidopsis during hypoxia (Zhan et al., 2018). The authors demonstrated that GSNOR1 *S*-nitrosation initiated the protein conformational modification exposing its AUTOPHAGY-RELATED8 (ATG8)-interacting motif (AIM) to autophagy machinery. Kovacs et al. (2016) proposed other, ROS-dependent regulation of GSNOR activity. They demonstrated the inhibition of GSNOR enzymatic activity as a result of *in vitro* treatment with H_2O_2 and *in vivo* paraquat application. The authors suggested that this regulation is related to oxidative modifications of Zn^{2+}-coordinating Cys-47 and Cys-177. Initiation of germination of apple embryos is also linked to increased ROS generation leading to temporal oxidative stress (Krasuska and Gniazdowska, 2012). Thus, we believe that ROS transient increase may acts as regulator of NO metabolism via e.g. modulation of GSNOR activity.

GSNOR activity is strictly linked to GSNO concentration. Data concerning the presence and concentration of GSNO in seeds during dormancy release and germination are unique. We have shown variations of GSNO level in axes of dormant and non-dormant apple embryos (Fig. 3). Higher level of GSNO in axes of non-dormant embryos correlates well with lower GSNOR activity (Fig. 2C). Our results are in agreement with those obtained for unstressed pepper (*Capsicum annuum* L.) plants. The lowest GSNOR activity in roots was linked to higher GSNO content (Airaki et al., 2011). Barroso et al. (2006) proposed that GSNO may induce GSNOR activity. However, our data indicate that during germination probably occurs other regulation of GSNOR activity since in axes of apple embryos a higher GSNO level is accompanied by a lower enzyme catalytic action.

GSH an abundant and stable metabolite plays a pivotal function not only as an antioxidant but participates in posttranslational modifications of proteins such as glutathionylation (Noctor et al., 2012). Increased GSSG level leads to decrease of GSH:GSSG ratio, resulting in more oxidised cellular redox state. Total glutathione pool and GSH content was higher in axes of non-dormant apple embryos (Table 1). Enhanced expression of genes coding gamma-glutamylcysteine synthetase and glutathione synthetase was observed after application of NO donors (Innocenti et al., 2007), suggesting impact of high NO level on regulation of glutathione synthesis. Moreover, in axes of dormant embryos around 12% of the total glutathione content was calculated for GSSG comparing to only 6% of GSSG from non-dormant plant material. High GSH:GSSG ratio in non-dormant tissue and lack of differences in GSSG concentration in axes of dormant and non-dormant embryos may be due to high GR activity in axes of stratified seeds (Table 2). The increase of total glutathione pool was observed in lupine (*Lupinus luteus* L.) embryonic axes before the onset of germination while afterwards a decrease of the glutathione content was noticed (Garnczarska and Wojtyla, 2008). Decline of the total glutathione was characteristic for barley embryos during first hours of post imbibition, which most probably was linked to intensive *S*-nitrosation of GSH. Nevertheless, the authors observed an increase of glutathione reduction after more than 24 h of post imbibition (Ma et al., 2016). Concentration of total glutathione and particularly GSH shows a close correlation with seed germination ability (Fontaine et al., 1995; Nagel et al., 2014). Nagel et al. (2014) demonstrated that artificial ageing and long-term seed storage leading to decline of viability and low germination rate, resulted in declining levels of total glutathione, GSH and increasing $E_{GSSG/2GSH}$ value. In axes of both dormant and non-dormant embryos $E_{GSSG/2GSH}$ was below −250 mV (Table 1), typical for seeds of high viability and high germination rate (Kranner et al., 2006; Nagel et al.,

Table 1
Glutathione (GSH and GSSG) concentration, GSH:GSSG ration and glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) in axes of dormant (control) apple embryos or in axes of non-dormant apple embryos isolated from stratified seeds. Values are average ± SD of 3–4 repetitions. Asterisks (*) indicate significance from the control at $P \leq 0.05$, based on student test.

Axes of embryos isolated from seeds	GSH (nmol g^{-1} FW)	GSSG (nmol g^{-1} FW)	GSH:GSSG	$E_{GSSG/2GSH}$ (mV)
Dormant (control)	531.6 ± 66.7	71.5 ± 18.8	7.4 ± 1.6	−256 ± 0.002
Non-dormant (stratified)	986.8 ± 57.2*	66.4 ± 23.6	14.9 ± 3.2*	−273 ± 0.005*

Table 2
Activity of GR and relative transcript level of genes coding GR in axes of dormant (control) apple embryos or in axes of non-dormant apple embryos isolated from stratified seeds. Expression of *GR* genes was normalized to *UCE* as a reference gene. Values are average ± SD of 3–4 repetitions. Asterisks (*) indicate significance from the control at $P \leq 0.05$, based on Student's test.

Axes of embryos isolated from seeds	GR activity (nmol mg^{-1} prot min^{-1})	Relative transcript level of GR genes	
		Plastidic	Cytosolic
Dormant (control)	0.70 ± 0.11	1.00 ± 0.01	1.00 ± 0.01
Non-dormant (stratified)	1.88 ± 0.35*	1.08 ± 0.10	1.42 ± 0.06*

2014). Our results are in agreement with the idea of Kranner et al. (2006) suggesting that changes of GSH or % of GSSG and particularly $E_{GSSG/2GSH}$ are universal markers for seed viability and germination.

GR activity prevents GSSG overaccumulation by reducing this molecule to GSH (Kranner et al., 2006). High GR activity seems to be essential for undisturbed seed germination as GR and other enzymatic antioxidants maintain sunflower (*Helianthus annuus* L.) seeds vigour (Bailly et al., 2002). Germination of apple embryos shortly pre-treated with HCN (Bogatek et al., 2003; Krasuska and Gniazdowska, 2012) or with NO (Krasuska and Gniazdowska, 2012) was accompanied with stimulation of GR activity. High GR activity was observed in germinating non-dormant barley caryopsis (Ma et al., 2016) and in embryonic axes of lupine (Garnczarska and Wojtyla, 2008). Therefore we were not surprised, that in axes of non-dormant apple embryos transcript levels of *GR* were higher as compared to the control (dormant) (Table 2). This points to a pivotal role of GR in seed dormancy release and in glutathione-NO regulation.

5. Conclusion

Our data have confirmed the importance of glutathione pool and its redox state, GSNOR and GSNO in seed dormancy removal. Increased GSH level and total glutathione pool in embryonic axes positively correlated with seed transition from dormant to ready-to-germinate state. GSNOR being a natural eliminator of GSNO seemed to be crucial for maintenance of cellular RNS level, although we suggested that GSNOR enzymatic activity (its decline) rather than alteration in the amount of the protein was linked to dormancy alleviation. These data matched well to elevated GSNO content in axes of stratified apple embryos. Formation of GSNO prevents over-accumulation of NO and GSNO participates in posttranslational protein modification (*S*-nitrosation), that may probably affect GSNOR activity. Therefore, alteration in GSNO and GSH content occurred during apple seed dormancy release and they were related to higher GR activity and gene transcription, regulating pool of these vital metabolites. Consequently, both enzymes GSNOR and GR could be considered as elements of biochemical mechanism for adjustment of intracellular NO level.

Conflicts of interest

The authors declare that they have no conflict of interest.

Authors contributions

K.C., K.O.-K. performed experiments, A.G., U.K., K.C. designed the work, U.K., K.C. A.G. analyzed experiments, U.K., A.G., K.C. wrote the manuscript. All authors have read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.02.026.

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