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Nitric oxide-polyamines cross-talk during dormancy release and germination of apple embryos

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ABSTRACT

Nitric oxide (NO) and polyamines (PAs) belong to plant growth and development regulators. These compounds play a key role in numerous physiological processes e.g. seed germination. Based on the suggestion of overlapping of NO and PAs biosynthetic pathways, we demonstrated a cross-talk of NO and PAs in regulation of embryonic dormancy release. The aim of the work was to investigate an impact of PAs (Put, Spd and Spm) or NO short-term fumigation on nitrite, urea, Arg and ornithine (Orn) content, NO synthase-like (NOS-like) and arginase activity in axes of apple (*Malus domestica* Borkh.) embryos during dormancy alleviation and at the stage of termination of germination *sensu stricto*. NO, Put/Spd induced dormancy breakage and germination of apple embryos corresponded to stimulation of urea cycle and high free Arg pool in seedlings roots. After two days of the culture Put and Spd stimulated Arg dependent NO formation, inhibition of which was observed after Spm application. Put or Spd application as well as NO short-term pretreatment of apple embryos influenced level of ubiquitin-conjugated proteins. Higher abundance of such modified proteins correlated well to the declined content of nitrated proteins, suggesting their important role in regulation of embryo germination. NO led to stimulation of embryos germination by increasing level of free PAs (mostly Put). While transcriptomic approach showed down regulation of Spm synthesis and up-regulation of Spm degradation by NO, confirming negative role of Spm over-accumulation in embryo dormancy removal. Our data clearly indicate positive relationship of NO-Put/Spd acting as dormancy removing factors.

1. Introduction

Reactive nitrogen species (RNS), including nitric oxide (NO) are bioactive compounds, which play essential roles as metabolic regulators of developmental processes also as messenger molecules involved in signal transduction [1,2]. The significant event in plant ontology is breaking of seed dormancy and the initiation of seed germination following by undisturbed seedling growth. These physiological stages are under the control of RNS, which cooperate with reactive oxygen species (ROS) and phytohormones [3,4]. NO concentration that values achieved the "nitrosative door" level enables seed germination. Results of many experiments indicated that different NO-donors stimulated seed germination of various plant species [5,6]. For a quite long time we focused our interest on NO mode of action during apple (*Malus domestica* Borkh.) embryos dormancy release. Apple embryos dormancy has been examined for a few decades [7]. Deep dormancy of apple is connected to an asynchronous development of cotyledons. Short-term pretreatment of dormant embryos with NO-donors breaks dormancy and stimulates seedling development [8–11].

In planta RNS are generated *via* non-enzymatic and enzymatic reactions [2,12]. The main non-enzymatic sources of RNS are nitrite ions (NO_2^-), which facilitate NO release at low pH or in the presence of reductants e.g. ascorbic acid [13]. There is no doubt, that nitrate reductase or nitrite reductase participates in NO enzymatic synthesis associated with reduction of NO_2^-. Though, both enzymes required specific conditions (low oxygen concentration) in the cells to catalyze liberation of NO [14]. The main enzyme involved in NO

Abbreviations: 3-NT, 3-nitro-tyrosine; Arg, arginine; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; Orn, ornithine; NO_2^-, nitrite; NO, nitric oxide; NOS-like, nitric oxide synthase-like; PAs, polyamines; PAO, polyamine oxidase; Put, putrescine; RNS, Reactive Nitrogen Species; ROS, Reactive Oxygen Species; SAMdc, *S*-adenosylmethionine decarboxylase; Spd, spermidine; SPDS, spermidine synthase; Spm, spermine; SPMS, spermine synthase.

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synthesis in mammalian cells is NO synthase (NOS), which exists in three isoforms [15]. Questionable is production of NO from arginine (Arg) catalyzed by NOS in higher plants, until now no homologous gene to mammalian NOS has been presented [1,16,17]. The existence of NOS protein of 45% homology to human protein has been shown only for the unicellular green alga *Ostreococcus tauri* [18]. Nevertheless, in higher plants NOS-like activity has been demonstrated in olive (*Olea europaea* L.), pea (*Pisum sativum* L.), sunflower (*Helianthus annuus* L.) [2,19,20], and apple [16,21]. NOS, in the presence of oxygen molecules catalyzes two-steps reaction of Arg oxidation to citrulline and NO. This enzyme of bimodal activity requires several cofactors: nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and 6R-tetra-hydrobiopterin (H_4B) [15,18]. The presence of H_4B in plant cells remains a matter of speculation; however OtNOS activity was noted with tetrahydrofolate [22]. One of the most important functions of RNS is modification of protein structure *via* *S*-nitrosylation or nitration [23–25]. Protein tyrosine (Tyr) nitration occurs, when a nitro (-NO_2) group is covalently bound to *ortho* position in the phenolic hydroxyl group of this amino acid. Addition of nitro group in the presence of nitro-oxidizing agents leads to formation of 3-nitro-tyrosine (3-NT) [23]. Just recently, we have demonstrated that during dormancy release of apple embryos nitrated proteins level has declined, and among these proteins were most likely presented seed biotin-containing proteins [16], serving as seed storage proteins subjected to degradation at the stage of germination. Ubiquitin, a heat-stable polypeptide has been shown to be abundant in all eukaryotic cells, including cotyledons of seeds [26]. Ubiquitin ligation to proteins targets them to degradation and this pathway occurs in many physiological processes, also seed germination [26,27].

Polyamines (PAs), which are regulators of plant growth and development, have been shown to participate in many physiological processes including seed germination [28], and reactions to stresses. However, various PAs have diverse impact on metabolic events [29–31]. PAs are low molecular weight polycationic, aliphatic, nitrogen-containing compounds of the positive charge which allows them binding to the various cellular macromolecules [32]. The major diamine in higher plants is putrescine (Put); spermidine (Spd) is a triamine, and spermine (Spm) is a tetraamine [33]. Arg, an important basic amino acid with a guanidine side chain, and ornithine (Orn) are precursors in Put biosynthesis [34]. This PA is synthesized through decarboxylation of Orn by Orn decarboxylase or by decarboxylation of Arg by Arg decarboxylase. Spd is formed from Put by addition of aminopropyl groups in reaction catalyzed by Spd synthase (SPDS). The addition of aminopropyl groups to Spd leads to Spm formation in reaction catalyzed by Spm synthase (SPMS). The aminopropyl groups come from decarboxylation of *S*-adenosylmethionine (SAM) by SAM decarboxylase (SAMdc) [30,31,33]. SAM is also involved in ethylene biosynthesis, since it acts as a substrate for formation of 1-aminocyclopropane-1-carboxylic acid (ACC), a direct precursor of this hormone [9,10,35]. NO-dependent stimulation of ethylene formation was demonstrated during germination of apple embryos [9]. Both ethylene and NO together to ROS controlled development of apple seedlings [10,11]. Catabolism of PAs depends on the activity of diamine oxidases (DAOs) and PAs oxidases (PAOs), which generates H_2O_2 [31].

NO-PAs link has been examined and discussed for a long time [30,36] but mostly in aspects of plant responses to stresses [29,31]. The evidence for PAs-NO cross-talk has come from observation of stimulated NO generation in Arabidopsis seedlings treated with PAs or in embryogenic culture of *Araucaria angustifolia* (Bertol.) Kuntze [37,38]. Since then, it was also demonstrated that PAs regulate level of carbonylated, nitrated and *S*-nitrosylated proteins in citrus (*Citrus aurantium* L.) plants exposed to salinity stress [39]. Nevertheless, little information is available on NO-PAs cross-talk during seed dormancy release and seed germination. Previously we have shown that Put stimulated apple embryo germination, accompanied by enhanced NO liberation from axes and roots [40].

Arg metabolism is linked to both NO and PAs synthesis. As we reported before, apple embryo germination in the presence of Arg resulted in an increased emission of NO from embryonic roots [40]. Arg, an amino acid of high N/C ratio is an important source of organic nitrogen, especially in seeds of various plant species, where represents 40–50% of total amount of this nutrient. Moreover, Arg is synthesized from Orn within the urea cycle [21,34]. Orn, a non-protein amino acid influences PAs synthesis and possible NO production from Arg. Arginase, a manganese metalloenzyme, catalyzes Arg conversion to urea and Orn [41]. Urea, a product of arginase activity is a source of organic nitrogen. High activity of arginase was observed especially during seed germination e.g. in cotyledons of bean or in excised embryonic axes of germinated soybean (*Glycina max* L.) seeds [42].

In this work we focused our interest on embryo dormancy release and germination *sensu stricto* in the context of NO-PAs cross-talk. We investigated impact of short-term pretreatment with NO or imbibition in PAs of apple embryos on arginase or NOS-like activity in extracts isolated from embryonic axes/roots during germination *sensu stricto*. We linked these activities to Arg, Orn and urea concentration, as well as content of nitrite. We measured free PAs level in axes of dormant and NO-pretreated embryos together to determination of transcript level of genes encoding PAO, SPDS, SPMS and SAMdc. As increased proteolytic activity is related to dormancy release [43], decrease of nitrated proteins level, and degradation of some reserve proteins is beneficial for undisturbed germination [16], we decided to investigate the pattern of ubiquitinated proteins and total content of nitrated proteins after PAs treatment of apple embryos. We demonstrated a strong proof for NO-PAs cross-talk during apple embryos dormancy release and germination *sensu stricto*.

2. Materials and methods

2.1. Plant material

Apple (*Malus domestica* Borkh., cv. Antonówka) dormant seeds were isolated from fruits (Andryka fruit producer Dąbrówka, Poland) and stored at 5 °C in glass containers until used. In all experiments seeds were imbibed in distilled water for 24 h and embryos were isolated for further analysis.

2.2. Embryos treatment with PAs

After 24 h imbibition in distilled water isolated embryos were placed on a filter paper moistured with water solution of hydrochloride forms of PAs: Put (0.2 mM), Spd (0.2 mM) or Spm (0.3 mM) in Petri dishes for 2 or 8 days [40]. Solutions of PAs were freshly prepared and changed every two days.

2.3. Embryos treatment with NO-donor or NO scavenger

After 24 h imbibition in distilled water isolated embryos were placed on filter paper moistured with 0.05 M K-phosphate buffer pH 7.0 in a glass container (0.5 L), enclosing a glass baker filled with a water solution of 20 mM $NaNO_2$ acidified with 0.2 M HCl, based on [13], and modified according to [10] (Fig. S1). Treatment was carried out at room temperature for 3 h, and after fumigation, embryos were washed twice in distilled water and placed on filter paper wetted with distilled water in Petri dishes. Control embryos

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