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meta-Tyrosine induces modification of reactive nitrogen species level, protein nitration and nitrosoglutathione reductase in tomato roots

Urszula Krasuska [a], Olga Andrzejczak [a], Paweł Staszek [a], Wojciech Borucki [b], Agnieszka Gniazdowska [a, *]

[a] Department of Plant Physiology, Warsaw University of Life Sciences–SGGW, Nowoursynowska 159, 02-776 Warsaw, Poland
[b] Department of Botany, Warsaw University of Life Sciences–SGGW, Nowoursynowska 159, 02-776 Warsaw, Poland

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ABSTRACT

A non-protein amino acid (NPAA) - *meta*-Tyrosine (*m*-Tyr), is a harmful compound produced by fescue roots. Young (3–4 days old) tomato (*Solanum lycopersicum* L.) seedlings were supplemented for 24–72 h with *m*-Tyr (50 or 250 μM) inhibiting root growth by 50 or 100%, without lethal effect. Fluorescence of DAF-FM and APF derivatives was determined to show reactive nitrogen species (RNS) localization and level in roots of tomato plants. *m*-Tyr-induced restriction of root elongation growth was related to formation of nitrated proteins described as content of 3-nitrotyrosine. Supplementation with *m*-Tyr enhanced superoxide radicals generation in extracts of tomato roots and stimulated protein nitration. It correlated well to increase of fluorescence of DAF-FM derivatives, and transiently stimulated fluorescence of APF derivatives corresponding respectively to NO and $ONOO^-$ formation. Alterations in RNS formation induced by *m*-Tyr were linked to metabolism of nitrosoglutathione (GSNO). Activity of nitrosoglutatione reductase (GSNOR), catalyzing degradation of GSNO was enhanced by long term plant supplementation with *m*-Tyr, similarly as protein abundance, while transcripts level were only slightly altered by tested NPAA. We conclude, that although in animal cells *m*-Tyr is considered as a marker of oxidative stress, its secondary mode of action in tomato plants involves perturbation in RNS formation, alteration in GSNO metabolism and modification of protein nitration level.

1. Introduction

Rhizosphere is a dynamic environment in which representatives of various species of plants and microorganisms compete for space, nutrients and water. Roots of plants, in addition to providing mechanical support, uptake of water and mineral nutrients, also play other specialized roles such as synthesis and secretion of various organic substances into the surrounding environment [1]. Compounds identified in root exudates are known to inhibit attack of herbivores or pathogens, stimulate the symbiotic interaction between plants and microorganisms, or influence growth of neighboring plants [2]. The phenomenon in which plants produce and release chemicals, affecting the development of other individuals is called allelopathy. Fescues plants (*Festuca*) comprises about 450 species typical for areas of moderate temperatures. They are used at golf courses, sports fields and lawns in North and South America and Europe [3]. Grasslands created with fescues do not grow other species e.g. clover (*Trifolium* L.), dandelion (*Taraxacum* F.H. Wigg.) or daisies (*Bellis* sp.). Almost 50 years ago it has been shown that the aqueous extracts from the dried roots of shrubs and tall fescue (*Festuca arundinacea* Schreb.) inhibited growth of roots of rape (*Brassica nigra* L.) [4]. A negative effect of fescues on seed germination, growth and development of birdsfoot trefoil (*Lotus corniculatus* L.) [5] or yield of red clover (*T. pratensis* L.) [6] was described. Allelopathic properties of fescues correspond to their root exudates. The main component of fescue roots' exudates is *meta*-Tyrosine (*m*-Tyr). The highest content of *m*-Tyr was recorded

Abbreviations: 3-NT, 3-nitrotyrosine; APF, 3′-(*p*-aminophenyl) fluorescein; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, 4-amino-5-methylamino-20,70-difluorofluorescein diacetate; GSNOR, *S*-nitrosoglutathione reductase; L-DOPA, dihydroxyphenylalanine; *m*-Tyr, *meta*-tyrosine; NO, nitric oxide; NPAA, non-protein amino acid; Phe, phenylalanine; RNS, Reactive Nitrogen Species; ROS, Reactive Oxygen Species; SNP, sodium nitroprusside.

* Corresponding author.
E-mail addresses: urszula_krasuska@sggw.pl (U. Krasuska), andrzejczakolga5@gmail.com (O. Andrzejczak), pawel.e.staszek@gmail.com (P. Staszek), wojciech_borucki@sggw.pl (W. Borucki), agnieszka_gniazdowska@sggw.pl (A. Gniazdowska).

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in *F. rubra* spp. Commutata and *F. rubra* spp. Rubra [7]. It occurs also in myrtle euphorbia (*Euphorbia myrsinites* L.). Moreover, *m*-Tyr is produced by certain strains of bacteria, and is a precursor for antibiotics such as mureidomycins, pacidamycins, and napsamycins [8,9].

m-Tyr belongs to the group of non-protein amino acids (NPAAs). In plant cells it is produced by the enzymatic hydroxylation of phenylalanine (Phe) (in *F. rubra*) or transamination of *m*-hydroxyphenylpyruvate (in *E. myrsinites*) [9]. In contrast, in animal cells, in oxidative stress conditions *m*-Tyr is accumulated as a result of non-enzymatic oxidation of Phe [10].

The mode of action of *m*-Tyr has not been fully recognized. The primary/basic mechanism of action of *m*-Tyr is thought to be associated with the incorporation of this NPAA into the structure of proteins resulting in formation of dysfunctional proteins [11,12]. *m*-Tyr is incorporated into proteins *in vitro* by a mechanism involving L-phenylalanine-tRNA synthetase [13]. Another hypothesis points that *m*-Tyr could be converted into dihydroxyphenylalanine (L-DOPA) and as such acts as potential phytotoxic agent [14].

Recently, a large body of literature has been published describing the changes in metabolism of reactive oxygen species (ROS) of the acceptor plant as potential mechanism of action of various phytotoxic substances, including allelochemicals (see Ref. [15] for review). Oxidative stress due to the excessive production of ROS and the lower efficiency of antioxidant system may be the first measurable effect of action of allelotoxins including *m*-Tyr. Further evidence has shown correlation between increase in the concentration of *m*-Tyr and excessive ROS production in the tissues of living organisms, especially animals, including humans [16]. In animal cells *m*-Tyr is considered as a marker of oxidative stress [17–19]. Accumulation of *m*-Tyr is related to such diseases as: Alzheimer's, atherosclerosis or diabetes [16,20]. Progression of neurodegenerative or cardiovascular illnesses is linked not only to ROS overproduction but also to disturbances in nitric oxide (NO) and other reactive nitrogen species (RNS) biosynthesis and metabolism [21,22]. Both in animal and plant organisms NO is recognized as a signaling molecule, action of which (despite other pathways) depends on modification of the structure of proteins [23–25]. Most of these modifications result from the actions of NO with ROS to produce reactive oxidants. The oxidants modify the proteins by *S*-nitrosylation of thiol groups or nitration of aromatic amino acids e.g. Tyr, by formation of 3-nitro Tyr (3-NT). Tyr nitration is the reaction of a nitrating agent with a Tyr residue of a target protein that lead to the addition of a nitro group (NO_2) in the *ortho* position of the phenolic hydroxyl group. The NO_2 group originates mainly from peroxynitrite ($ONOO^-$), which is generated in reaction of superoxide anion ($O_2^{\bullet -}$) and NO [24,26].

Cellular concentration of NO depends on its biosynthesis and scavenging [26,27]. Glutathione (GSH), despite its action as an antioxidant, reacts also with NO leading to formation of nitrosoglutathione (GSNO). This molecule is a major cellular bioreservoir of NO. The GSNO pool is regulated by activity of GSNO reductase (GSNOR), formerly known as glutathione-dependent formaldehyde dehydrogenase (FALDH; EC 1.2.1.1), which converts GSNO into oxidized form of glutathione (GSSG) and ammonia [28]. According to formal enzyme classification, GSNOR belongs to a family of Zn-dependent class III alcohol dehydrogenases (ADH3; EC 1.1.1.1). GSNOR has important role in the regulation of GSNO content in plants during development and under stress conditions (see Ref. [28] for review). Xu et al. [29] suggested even that GSNOR facilitates multiple homeostatic and stress adaptation processes in plants.

At present, there is no data evaluating disturbances in ROS and RNS level/metabolism as the mechanism of phytotoxicity of *m*-Tyr in plant tissues. Recently we have shown that L-canavanine's (another toxic NPAA) mode of action is associated with accumulation of ROS and alterations in RNS level coupled to enhanced protein carbonylation [30,31]. Taking to account that *m*-Tyr is considered as an indicator of oxidative stress, we suspect that its supplementation may impact also RNS metabolism and content, and in consequence influence protein nitration, one of RNS-regulated protein posttranslational modifications (PTMs). Protein nitration is as a reliable marker of nitrosative stress and seems to be typical for plants responding to various unfavorable conditions of both biotic (pathogens) or abiotic nature (see Refs. [32,33] for review).

Thus, one of the aim of the work was to link the phytotoxic effect of *m*-Tyr on root growth of tomato seedlings to modification of RNS level and tissue distribution accompanied by determination of 3-NT content. Due to the role of GSNOR, which is suggested to be a key element in the interplay between the ROS and RNS metabolisms [34], we pointed our interest at its activity, protein level and gene expression. Even if (as reviewed by Petřivalský et al. [28]) GSNOR activity and expression varied depending on environmental conditions, no simple correlation between application of oxidative agents or ROS (H_2O_2 or paraquat) and GSNOR activity, gene expression or protein level are confirmed [35,36]. The experimental model, we are using for investigation of regulation of elongation growth of roots [30,31,37,38] seems to be very convenient to build up a general idea of mode of action of *m*-Tyr, with a special attention pointed at its putative, secondary effect related to RNS metabolism.

2. Materials and methods

2.1. Plant material

Seeds of tomato (*Solanum lycopersicum* L. cv. Malinowy Ożarowski) were germinated in water at 20 °C in darkness for 3 days. After this period seedlings of equal roots' length (5 mm) were separated and transferred to Petri dishes (φ 15 cm) containing filter paper moistened with distilled water (control) or *m*-Tyr (Sigma-Aldrich) dissolved in distilled water pH 7.5. Culture of control seedlings and seedlings treated with *m*-Tyr on Petri dishes was performed in a growth chamber at 23/20 °C, 12/12 h day/night regime for 24 h or 72 h.

The concentration of *m*-Tyr (50 μM) reducing root length to 50% of the control, was accepted as IC_{50}. The concentration (250 μM), which completely inhibited elongation growth of roots, was accepted as IC_{100}.

2.2. Morphological studies of roots

For measuring root morphology the automated WinRHIZO system (Regent Instruments, Québec, Canada) have been used. Parameters of roots: length, diameter, total area, volume and number of root tips were analyzed. Root growth and development was monitored at time point of *m*-Tyr treatment at 24 h and 72 h by taking digital images of roots with a WinRhizo root scanner, and typical images are shown.

2.3. Test of cell viability

Viability of tomato root cells was determined using Evans blue staining, increase in tissue staining corresponds to decline in viability. Whole control seedlings or seedlings treated with *m*-Tyr (50, 250 μM) for 24 h and 72 h were incubated in 0.25% solution of Evans blue for 30 min at room temperature. Then seedlings were washed twice in distilled water and roots were isolated, weighed and homogenized in 1 ml of 1% solution of sodium dodecylsulphate