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Involvement of NR and PM-NR in NO biosynthesis in cucumber plants subjected to salt stress

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ABSTRACT

Nitrate reductase (NR) mainly reduces nitrate to nitrite. However, in certain conditions it can reduce nitrite to NO. In plants, a plasma membrane-associated form of NR (PM-NR) is present. It produces NO_2^- for nitrite NO/reductase (Ni-NOR), which can release NO into the apoplastic space. The effect of 50 mM NaCl on NO formation and the involvement of NR in NO biosynthesis were studied in cucumber seedling roots under salt stress. In salt-stressed roots, the amount of NO was higher than in control. The application of tungstate abolished the increase of NO level in stressed roots, indicating that NR was responsible for NO biosynthesis under the test conditions. The involvement of other molybdoenzymes was excluded using specific inhibitors. Furthermore, higher cNR and PM-NR activities were observed in NaCl-treated roots. The increase in NR activity was due to the stimulation of *CsNR* genes expression and posttranslational modifications, such as enzyme dephosphorylation. This was confirmed by Western blot analysis. Moreover, the increase of nitrite tissue level in short-term stressed roots and the nitrite/nitrate ratio, with a simultaneous decrease of nitrite reductase (NiR) activity, in both short- and long-term stressed roots, could promote the production of NO by NR in roots under salt stress.

1. Introduction

Nitric oxide (NO) is a small, gaseous molecule, widely recognized as an important signal generated in plants in response to biotic and abiotic stressors [1,2][reviewed in 1,2]. Furthermore, NO is involved in the range of normal developmental and physiological processes from germination to fruit ripening and senescence. The function of nitric oxide in stomatal closure, plant root formation and programmed cell death has been well established and reviewed [3,4]. According to the present knowledge, NO signaling at the molecular level involves the modulation of gene expression and various protein modifications [5]. These include transfer of NO from nitrosothiol (GSNO) and binding to the sulfhydryl group of a critical cysteine residue (S-nitrosylation) and/or tyrosine residue nitration [6,7].

Biosynthesis of NO in plants is very complex and many different enzymatic and non-enzymatic pathways have been described. Non-enzymatic NO generation can occur in the acidic space of the apoplast in the presence of ascorbic acid or other reductants [8]. Enzymatic pathways of NO production are generally classified as oxidative or reductive [2,9]. Oxidative pathways involve nitric oxide synthase-like activity (NOS-like activity). Although NOS-like activity in plants requires the same cofactors as in mammals and exhibits similar sensitivity on mammalian NOS inhibitors, a plant enzyme similar to animal-type NOS has not yet been identified [10]. In *Arabidopsis thaliana*, and recently in cucumber, NO-associated 1 protein (AtNOA1 and CsNOA1, respectively) has been recognized as a protein associated with NO production [11,12]. However, both enzymes are not a mammalian-type of nitric oxide synthase, as they show different co-factor requirements [10,13]. An alternative oxidative route of NO production in plant tissues has been proposed that involves polyamine and hydroxylamine metabolism [7,14].

Nitrate reductase located in the cytoplasm (cNR) is the most important reducing and enzymatic source of NO in plants. The primary function of cytosolic NR is the reduction of nitrate to nitrite during nitrate assimilation in plants. However, it is well documented that NR can also reduce nitrite ions to NO with NADH as an electron donor in specific cell conditions [2,9]. NR-mediated NO synthesis requires a relatively low nitrate concentration and a high nitrite concentration, so that NR affinity for nitrite is lower than that for nitrate ions ($K_{mnitrate}$ = 40 μM, $K_{mnitrite}$ = 100 μM) [3,15]. According to common knowledge, NR activity is subject to complex regulation at multiple levels [16]. It appears that posttranslational, reversible modifications of

Abbreviations: cNR, cytoplasmic nitrate reductase; DAF, diaminefluorescein; Ni-NOR, nitrite-nitric oxide reductase; NiR, nitrite reductase; NO, nitric oxide; NOS, nitric oxide synthase; PM-NR, plasma membrane bound nitrate reductase

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the NR protein, causing changes in NR catalytic activity, are very important for NO production in response to different factors [15]. The phosphorylation of NR in the conserved Ser residue and subsequent binding of the 14-3-3 inhibitor protein in the presence of divalent cations lead to enzyme inactivation. On the other hand, dephosphorylation of NR and consequent dissociation of 14-3-3 increase NR activity [16]. Cytosolic NR-mediated NO production has been reported in response to fungal pathogen attack [17], osmotic stress [18], water stress [19] and hypoxia [20].

Reductive, nitrite-dependent NO production also occurs in the membrane fraction. It was demonstrated that a plasma membrane-bound enzyme, nitrite-nitric oxide reductase (Ni-NOR), was responsible for NO generation in the apoplast, especially in roots [21]. The plasma membrane Ni-NOR requires nitrite, which is probably provided by plasma membrane-bound nitrate reductase (PM-NR), and both enzymes may be closely associated in the root cell membrane. PM-NR does not generate NO itself, but can influence Ni-NOR-mediated NO synthesis by nitrite production [21]. Although PM-NR catalyzes the reduction of NO_3^- to NO_2^-, it probably differs from cytosolic NR, as it can use succinate as an additional electron donor. However, similar sensitivity of PM-NR to Mg^{2+} ions has also been reported [22].

In addition to NR and PM-NR, other molybdoenzymes, i.e., cytoplasmic aldehyde oxidase (AO) and peroxisomal xanthine oxidoreductase (XOR) have been proposed to generate NO by nitrite ion reduction [23]. NO production catalyzed by these enzymes occurs only under limited oxygen conditions [24]. However, there is little evidence of their common role in plants [17]. NO biosynthesis through the nitrite reduction pathway may also occur in the mitochondrial inner membrane. It has been shown that the mitochondrial respiratory chain is able to produce NO via nitrite reduction by cytochrome c oxidase and/or reductase in plant cells exposed to hypoxia [2,17].

Salinity is a major environmental factor that significantly affects plant growth. This is a complex stress that imposes ionic toxicity, osmotic stress as well as oxidative damage of plant cells [25]. It has already been reported that NO plays an important role in improving plant tolerance to NaCl treatment, since the application of the artificial NO donor, sodium nitroprusside (SNP), increases NaCl tolerance, and treatment with the NO scavenger, PTIO, induces NaCl sensitivity in *Arabidopsis* and reed plants [26,27]. It has been shown that NO improves the activity of the plasma membrane proton pump, H^+-ATPase, which is a critical component in plant response to salinity [28]. NOS-like activity and AtNOA1 have been reported as a potential NO source during NaCl treatment. However, NR-mediated NO production in response to NaCl treatment may also be considered. The involvement of NR in NO generation during osmotic stress has been recently demonstrated [18]. Moreover, NR-mediated NO acts as signaling molecule in ABA-induced stomata closure [29].

The aim of the present study was to investigate the effect of NaCl treatment on the activity of cytoplasmic and plasma membrane-bound forms of nitrate reductase in cucumber roots. Both enzymes are directly or indirectly involved in NO generation, but there is insufficient data to confirm whether they are associated with NO production under salt stress. Concentrations of nitrate and nitrite as well as nitrite reductase activity were analyzed in salt-stressed roots in order to determine cell conditions favoring NO generation by NR.

2. Material and methods

2.1. Plant material

Experiments were conducted on 6-day-old cucumber seedlings (*Cucumis sativus* L. Wisconsin). Seeds were surface sterilized and germinated in darkness on wet filter paper at 25 °C for 48 h. Then they were transferred to a nutrient solution, pH 6.2, containing 1.7 mM KNO_3, 1.7 mM $Ca(NO_3)_2$, 0.33 mM KH_2PO_4, 0.33 mM $MgSO_4$ and microelements: 25 μM ferric citrate, 3.33 μM $MnSO_4$, 1.7 μM H_3BO_3, 0.3 μM $CuSO_4$, 0.003 μM $ZnSO_4$, 0.017 μM Na_2MoO_4 (control). The nutrient solution was continuously aerated. The plants were treated 1 day (short-term) or 6 days (long term) with 50 mM NaCl before collection for research. NaCl was added with nutrient solutions. At the same time in control plants nutrient solutions were replaced with the new one (1 or 6 days before collection). The growth conditions were as follows: 16-h photoperiod with light intensity 180 μmol m^{-2} s^{-1} at 25 °C/22 °C during day/night. Roots for every analysis were harvested after 4 h of starting of illumination.

2.2. Endogenous NO detection

Nitric oxide production in root tissues was detected by imaging the NO-specific fluorescence using the fluorescent NO indicator dye DAF-2D (5,6-diaminofluorescein diacetate). Roots of cucumber seedlings were briefly excised from the plant and incubated for 10 min at room temperature in the dark in 20 mM Hepes-KOH, pH 7.4 containing 10 μM DAF-2D. To remove excess fluorophore from the surface, roots were washed for 15 min in fresh 20 mM Hepes buffer renewed twice. NO-associated fluorescence was detected with the fluorescent microscope Zeiss Axio Image M2 using unchanged parameters for every measurement. For fluorescence observation a Tag-YFP filter with emission of 524 nm was used. The intensity of green fluorescence in the images was analyzed using Adobe PhotoshopCC software and expressed as the average number of pixels in green channel on a scale ranging from 0 to 225.

2.3. Preparation of cytosol extract and plasma membrane isolation

All steps were performed at 4 °C. Cucumber root tissue was homogenized in a chilled mortar with addition of 1 mM PVPP and extraction buffer 25 mM BTP-MES (pH 7.5) containing 330 mM sorbitol, 5 mM EDTA, 5 mM DTT, 0.5 mM PMSF, 0.2% BSA, then filtered through 4 layers of muslin and centrifuged at 18 000*g* for 10 min at 4 °C. The pellet was discarded and the supernatant was used for determination of cytosol NR activities and for isolation of the plasma membrane fraction. PM vesicles were isolated using a two-phase system according to the method of Larson [30] with some modifications of Kłobus [31]. The supernatant was centrifuged again at 80 000*g* for 30 min. The obtained pellet was suspended in 5 mM BTP-MES (pH 7.5) containing 330 mM sorbitol, 5 mM KCl and 0.1 mM EDTA and loaded into a two-phase system composed of 6.2% (w/w) Dextran T500, 6.2% (w/w) polyethylene glycol 3350, 330 mM sorbitol, 5 mM BTP-MES (pH 7.5) with 5 mM KCl and 0.1 mM EDTA. After centrifugation at 500*g* for 5 min, the upper phase was collected, loaded on a fresh lower phase prepared as described by Larson et al. [32] and centrifuged again to increase the upper fraction purity. The upper phase was then collected and diluted five-fold in 5 mM BTP-MES (pH 7.5) with 330 mM sorbitol, 5 mM KCl and 0.1 mM EDTA. After centrifugation at 80 000*g* for 30 min the pellet containing the PM vesicle fraction was resuspended in the same buffer and used for PM-NR activity measurement. PM fraction purity was checked by measuring activities of cytosol marker enzymes, alcohol dehydrogenase (ADH) and phosphoenolpyruvate carboxylase (PEPC) according Chung and Ferl [33], and Spalding and Edwards [34] respectively.

Protein content of PM fraction was determined according to Bradford [35] using BSA as a standard.

2.4. Nitrate reductase assay

NR activity was measured in the absence of Mg^{2+} (total NR activity, NR_{total}) and/or in the presence of Mg^{2+} (actual activity, NR_{act}) according to Kaiser and Huber [36] with some modifications [37]. The reaction mixture contained 50 mM Hepes-KOH (pH 7.5) with 5 mM EDTA or 5 mM $MgCl_2$ (respectively for total or actual NR activity), 10 mM KNO_3 and 1% Triton X100 for PM-NR determinations. The