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Short communication

Nitric oxide is involved in ethylene-induced adventitious root development in cucumber (*Cucumis sativus* L.) explants

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ABSTRACT

Both ethylene (ETH) and nitric oxide (NO) are endogenous signaling molecules that regulate plant adventitious root development. The relationship between ETH and NO in that process remains poorly understood. In this report, our results show that the effects of ETH on adventitious rooting in cucumber (*Cucumis Sativus* L.) explants were dose-dependent, and 0.5 μM ETH donor ethrel treatment was the most effective concentration to promote adventitious rooting. The positive effect of ETH could be reversed by NO scavenger 2-(4-carboxy-2-phenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), nitric oxide synthase (NOS) inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) or nitrate reductase (NR) inhibitor NaN_3, indicating that the ETH-induced response, at least partially, was NO-dependent. Meanwhile, the exposure of cucumber explants to ETH up-regulated NOS and NR activity and their gene relative expression. Moreover, molecular evidence indicated that ETH improved the expression of genes involved in adventitious root development, including DNAJ-like gene (*CsDNAJ-1*) and calcium-dependent protein kinase genes (*CsCDPK1* and *CsCDPK5*). However, the effects of ETH were inhibited by c-PTIO, L-NAME or NaN_3. The results indicate that NO plays crucial role in the adventitious root development induced by ETH.

1. Introduction

Adventitious roots, which are stimulated by the removal of root tissue, originate from plant stems and leaf petiole, as well as from non-pericycle tissue. Adventitious root development is a complex process induced by many internal and external signals including plant hormones and signaling molecules (Pagnussat et al., 2003, 2004). Auxins have been shown to be the major growth-promoting hormones for adventitious root development (Bai et al., 2012). Carbon monoxide (CO) (Xu et al., 2006), nitric oxide (NO), hydrogen peroxide (H_2O_2) (Liao et al., 2009, 2011; Liao et al., 2012a,b) and Hydrogen gas (H_2) were reported to be involved in adventitious rooting (Lin et al., 2014). Several studies have focused on understanding molecular processes associated with the initiation and development of adventitious root in different plant species (Lanteri et al., 2006; Xuan et al., 2008). Thus, more studies on this topic will help us understand signal transduction and its mechanism during plant adventitious rooting.

As a gaseous phytohormone, ethylene (ETH) in most plant tissues and cell types play important role in diverse developmental processes including seed germination, flower and leaf senescence, and fruit ripening (Abeles et al., 1992). As a modulator, it is also involved in response to biotic and abiotic stimuli and stresses in plants, such as pathogen attack, flooding, chilling, and mechanical damage (Johnson and Ecker, 1998; Bleecker and Kende, 2000). Moreover, ETH promoted root hair formation, while ETH biosynthesis inhibitor aminovinylgly-cine (AVG) abolished root hairs (Pitts et al., 1998). ETH also promoted the initiation of lateral root primordia derived from the pericycle, as well as the emergence and elongation of lateral root (Ivanchenko et al., 2008). Arabidopsis *CULLIN3* genes modulated the emission of ETH and regulated primary root growth via a novel ethylene-dependant pathway (Thomann et al., 2009). ETH production induced by flooding stimulated adventitious root emergence in wetland plants (Steffens et al., 2006). Therefore, these reports provide evidence that ETH pathway may act on root development and growth atdifferent levels.

NO, an easily diffused and almost universal signaling molecule, is involved in regulating plants responses to various stimuli and

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development including senescence (Liao et al., 2013), drought (Wodala et al., 2008; Liao et al., 2012a,b), seed germination (He et al., 2014), flowering (Zeng et al., 2011) and stomatal closure (Daszkowska-Golec and Szarejko, 2013). Several researchers have reported that low concentrations of NO promotes seed germination (Jacobsen et al., 2013) and root formation (Liao et al., 2009). A number of studies have found that NO might be produced by plant extracts incubated with nitric oxide synthase (NOS) substrate arginine. Therefore, a NOS-like activity does exists in plants (Asai and Yoshioka, 2009; Corpas et al., 2006). Nitrate reductase (NR) has been shown to take part in NO production in some physiological activities (Bellin et al., 2013). Our previous results showed that NOS and NR were involved in the NO production in marigold (*Tagetes erecta* L.) explants during adventitious rooting (Liao et al., 2009).

It is well known that ETH and NO function as important signaling molecules involved in plant adventitious rooting. Previous results from our laboratory have shown that NO might extend the postharvest life of cut rose flowers by down-regulating ETH production (Liao et al., 2013). However, relatively little work about the "cross-talk" between ETH and NO during adventitious root development has been done. Therefore, the objective of the present study was to investigate the roles and relationship of ETH and NO during adventitious rooting process in cucumber.

2. Materials and methods

2.1. Material and growth conditions

Cucumber (*Cucumis sativus* L. var. 'Xinchun 4') seeds were purchased from Gansu Agricultural Institute (Lanzhou, China). The seeds were surface-sterilized in 5% sodium hypochlorite for 10 min, washed with water, germinated in petri dishes with double- layer filter paper moistened with distilled water. The seeds were put in an electronic growing chamber at 25 ± 1 °C for 5 days with a 14-h photoperiod (photosynthetically active radiation = 200 μmol s^{-1} m^{-2}). Primary roots of 5-day-old seedlings were removed and the cucumber explants were then maintained under the same conditions of temperature and photoperiod for 5 days under different treatments as indicated below. Data were taken on root number per explant and root length.

2.2. Treatments and experimental design

Cucumber explants were placed into petri dishes with 10 mL distilled water, or 10 mL of various concentration of ETH donor 2-chloroethylphosphonic acid (ethrel) (0, 0.1, 0.5, 1, 10, 50 μM). The following chemicals were added alone or together with ethrel: (1) 50 μM NO donor sodium nitroprusside (SNP; Sigma, USA); (2) 200 μM 2-(4-carboxy-2-phenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO; Sigma, USA); (3) 20 μM *N*-nitro-L-arginine methyl ester (L-NAME; Zhongtai Chemical Co., Ltd., Shanghai, China NAME, Sigma, USA); (4) 20 μM NaN_3 (Zhongtai Chemical Co., Ltd., Shanghai, China) was administered to explants. The concentration of these chemicals was selected based on the results of preliminary experiment in our laboratory. The treatments were arranged in a completely randomized design in three replicates. Each experimental unit consisted of ten individual explants from which data was taken.

2.3. Determination of endogenous NO content

The endogenous NO level was analyzed according to the method of Liao et al. (2011). Half of a gram (0.5 g) of hypocotyls were frozen in liquid nitrogen, then ground in a mortar and homogenized in 3 mL of 50 mM ice-cold acetic acid buffer pH 3.6, containing 4% (w/v) zinc diacetate. The homogenates were centrifuged at 10,000 × *g* for 15 min at 4 °C and the supernatants were collected. The pellets were washed using 1.0 mL of the above extraction buffer and centrifuged. Activated charcoal (0.1 g) was added to the supernatant, which was then filtrated and the absorbance was determined at 540 nm. The NO content was calculated by comparison with a standard curve of $NaNO_2$.

2.4. Determination of NOS activity

NOS activity was analyzed according to Murphy and Noack (1994) with some modifications. Two (2) g of fresh cucumber explants samples were homogenized in 5 mL of homogenization buffer (50 mM triethanolamine hydrochloride, pH 7.5, containing 0.5 mM EDTA, 1 μM leupeptin, 1 μM pepstatin, 7 mM glutathione, and 0.2 mM phenylethylsulfonyl fluoride). After centrifugation at 10,000 × *g* for 20 min, the supernatant was collected and re-centrifuged at 100,000 × *g* for 45 min at 4 °C. This supernatant was used for NOS determinations. Activity was obtained by a hemoglobin assay.

2.5. Determination of NR activity

NR activity was analyzed according to Rosales et al. (2012). Cucumber explants were homogenized in a medium containing 5 mM EDTA, 5 mM GSH, 1% (w/v) casein, 0.1% (w/v) insoluble PVP and 50 mM HEPES (pH 7.5) and centrifuged for 15 min at 17,000 × *g*. The assay mixture containing 200 μmol KNO_3, 0.2 μmol NADH and 100 μL of the homogenate was used to measure NR activity. The reaction was stopped by 50 μL 1 M zinc acetate after incubation at 30 °C for 20 min. The mixture was centrifuged 5 min at 7600 × *g* and the supernatant was used to determine nitrite production by reading the absorbance at 540 nm after the addition of 1% sulphanilamide in 1.5 M HCl and 0.01% N-(1-naphthyl)-ethylene diammonium dichloride.

2.6. Semi-quantitative RT-PCR analysis

Total RNA was extracted from about 100 mg (fresh-weight) excised cucumber hypocotyls, reverse-transcribed and cDNA abundance were measured by semi-quantitative RT-PCR. The cDNA was amplified using the following primers: for *Action* (accession number DQ641117), forward CCCATCTATGAGGGTTACGCC and reverse TGAGAGCATCAGTAAGGTCACGA; for *NOS* (accession number XM_004139725.1), forward CTACGGCACCAGCATAAGAAGA and reverse ACACGGTTGTCCATAAAATGATAA; for *NR* (accession number JQ692875.1), forward AAACCCTACATCCTTCACTCTCG and reverse GGTCCATTGCCATTTCTCTTCT; for *CsDNAJ-1* (accession number X67695), forward GACCACTCTCCACGATGTCAAC and reverse ATCAATGTGTTATGGCGGTAGC; for *CsCDPK1* (accession number AJ312239), forward GGAGTTGGAAGGAGGACGATG and reverse TGAGATTTAGCAGTAAGGACGC; for *CsCDPK5* (accession number AY02785), forward ATGAGGAAAGGCAATCAGGAAT and reverse AAAGAAGCACATAAAATCAAGCAGA; To standardize the results, the relative abundance of Actin was determined and used as the internal standard.

The cycle numbers of the PCR were adjusted for each gene to obtain visible bands on agarose gels. Aliquots of the PCR reactions were loaded on 2% agarose gels with the use of ethidium bromide (EB). Specific amplification products of the expected size were observed, and their identities were confirmed by sequencing.

2.7. Real-time RT-PCR analysis

Total RNA was isolated from 100 mg (fresh-weight) of excised cucumber hypocotyls by grinding with mortar and pestle in