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Inhibition of nitric oxide synthesis delayed mature-green tomato fruits ripening induced by inhibition of ethylene

Yang Yang[a], Yanyan Zheng[a], Can Liu[a], Lin Chen[a], Junfei Ma[c], Jiping Sheng[b], Lin Shen[a,*]

[a] College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, 100083, China
[b] School of Agricultural Economics and Rural Development, Renmin University of China, Beijing 100872, China
[c] College of Life Science, Qingdao Agricultural University, Qingdao, 266000, China

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ABSTRACT

Ethylene is a key molecule to quality preservation during fruit ripening. The ethylene biosynthesis is regulated partly by nitric oxide (NO), a signal molecule involved in various physiological reactions. In order to investigate the effect of NO on key factors of ethylene biosynthesis, mature green tomatoes (*Solanum lycopersicum* Mill. cv. Lichun) were treated with the NO synthesis inhibitor L-Nitro-arginine methylester (L-NAME). The results showed that L-NAME decreased endogenous ethylene release and delayed the breaker stage of fruits. In the process, the activities of 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxide (ACO) were inhibited in accordance with the down-regulation of *SlACS2/4* and *SlACO1/3* genes. In addition, the expression of calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK) genes, *SlCDPK1/2* and *SlMAPK1/2/3*, was delayed or reduced. These results suggest that protein phosphorylation is involved in the ethylene biosynthesis reduction induced by L-NAME and that this lead to a delay in tomato fruit ripening.

1. Introducion

Ethylene is the signal molecule, which affects respiration rate, regulates ripening–senescence, and mediates disease resistance in mature fruits. Thus, metabolism regulation, quality control, and shelf life extension depend on ethylene regulation in post-harvest fruits.

NO is an important signal molecule that regulates diverse physiological processes in phylogenetically distant species, including biotic-abiotic stress responses and growing-development. Recently, the effect of NO on postharvest control has also received attention. According to general view, exogenous NO delays fruit ripening and senescence due to the downregulation of endogenous ethylene. Exogenous NO reduces the low-temperature chilling injury of mango fruit and Japanese plums, and inhibits endogenous ethylene production (Hu et al., 2014; Singh et al., 2009). Pre-harvest treatment with sodium nitroprusside (SNP), which is NO donor, delays the accumulation of ethylene in 'Golden Delicious' apple fruit (Deng et al., 2013). However, contrary results were reported in which nitric oxide played a physiological role in the positive regulation of ethylene production and up-regulation expression of the key gene for ethylene biosynthesis. A study on how ethylene promotes the germination of Arabidopsis seeds under salinity stress showed that SNP treatment influences the expression of the *AtACS2* gene (Lin et al., 2013). Ethylene positively regulates nucellus-programmed cell death in *Sechium edule*, and its synthesis can be induced in part by NO (Lombardi et al., 2012). In cucumber plants, purified sedimentary humus acid increases NO accumulation in the root, followed by a concomitant increase in ethylene production in the same organ (Mora et al., 2012). In addition, treatments with NO synthesis inhibitors produce the opposite effect. So, NO produced from mammalian-like nitric oxide synthase (NOS) promotes the accumulation of UV-B-induced ethylene in maize seedlings, while NOS inhibitor exerts the opposite effect (Wang et al., 2006). But the research of NO synthesis inhibitor in fruits ripening and senescence was lack. It is necessary to study on effect of NO synthesis inhibitor for ethylene production, but relevant report is quite lacking.

Pattern of NO synthesis inhibitor include two major classes, inhibitor of NOS and inhibitor of nitrate reductase (NR), because NO is synthesized mainly through NOS and NR in plants (Mur et al., 2013). NOS reaction was initially discovered in mammals, and includes two types, constitutive and inducible (Furchgott, 1995). Although the NOS gene and protein have not yet been acquired in plants, sufficient experimental evidence proves the existence of plant NOS (Pedroso and Durzan, 2000). When the inhibition of the

* Corresponding author.
E-mail address: shen5000@cau.edu.cn (L. Shen).

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photosynthetic electron transport chain leads to nitrite accumulation, NR catalytic the synthesis of NO (Yamasaki and Sakihama, 2000). The NR is an inducible enzyme that is mainly present in leaves and roots of plants (Kaplan et al., 1974), however NO signals are produced in nearly all plant tissues and organs (Durner and Klessing, 1999). Thus, the synthesis of NO in fruits trough NOS deserves considerable attention. In a previous study we found that NOS is a key enzyme regulating the NO production in tomato fruits related to pathogen resistance (Zheng et al., 2011). L-Nitro-arginine methylester (L-NAME) and Nomega-Nitro-L-arginine (L-NNA) are well-known competitive inhibitors of NOS in animals, and their mechanisms of inhibition are similar (Awooda et al., 2013; Hobbs and Gibson, 1990). It has been confirmed that L-NAME and L-NNA inhibited NO synthesis in tomato cell suspensions and in tomato fruits (Zheng et al., 2011; Foresi et al., 2007).

Protein phosphorylation like cascades plays an important role in responses to various biotic and abiotic stresses (Davis, 1993; Sebastia et al., 2004) Morever, signalling molecule NO cross with pathways of protein phosphorylation (Lanteri et al., 2006; Pagnussat et al., 2004). Recent study showed that protein phosphorylation was regulated by semiochemicals including NO and involved in activation of key proteins about ethylene biosynthesis (Wen, 2014).

On the basis of the findings above, it is crucial to analyze the effects of NOS inhibitor on ethylene content and protein phosphorylation in fruits. Tomato fruit characteristically follows a pattern controlled by ethylene, and the mature-green stage is a key period in regulating fruit ripening. In the present work, effect of decreasing NO content by NO synthesis inhibitor L-NAME on ethylene biosynthesis and related gene about protein phosphorylation in postharvest mature-green stage tomato fruit were investigated, aiming at gaining a better understanding of the relationship between ethylene and NO in the ripening process of fruits.

2. Materials and methods

2.1. Plant material and treatments

Tomatoes of an early-maturing variety were grown in a greenhouse at Beijing(latitude 39°54′N; longitude 116°25′E) under conventional conditions using standard cultural practices. Flowers were tagged at pollination, and fruits were harvested 38–43 days after pollination (the Mature Green stage of fruit development). After harvesting, fruits were transported to the laboratory immediately and sorted to exclude those with obvious defects, inappropriate size, mechanical wounds, and disease.

Fruit were infiltrated in either water (control) or 0.1 mM L-NAME (LC Laboratories, Woburn, MA, USA), solutions under a pressure of −35 kPa for 0.5 min, then kept in the solution under air pressure for 2 min and then air dried. All fruit were stored at 25 ± 1 °C with 85–90% RH. Three replicates were conducted in above control and treatment.

2.2. Measurement of the statistical of ripeness and ethylene production rate

Twenty fruits chosen randomly in each control and L-NAME treatment were used to carry out the statistics of fruit maturity and determine ethylene production rate.

2.2.1. The statistical of the maturity index

Ripeness of the whole fruit was assessed on the basis of external visual appearance, with 1 = mature-green, 2 = breaker, 3 = turning, 4 = pink, 5 = light-red, and 6 = red-ripe (USDA, 1975). The maturity index on the external visual appearance of each treatment adopted the formula $\Sigma S_i/N$, where S_i is the score of each fruit based on external visual appearance and N is the total amount of each treatment group.

2.2.2. Measurement of ethylene production rate

Ethylene production rates of fruit were determined during 10d of the ripening period at 25 ± 1 °C, and measured using a gas chromatograph(GC-4000, East & West Analytical Instruments, INC., Beijing, China)fitted with a 2-m long, stainless steel column, a methane conversion oven, and a flame ionization detector (FID). The column temperature was 70 °C, the injection temperature was 150 °C, and the carrier gas was N_2 with a rate of 40–50 mL min^{-1}. The air and H_2 pressures used in the FID system were controlled at 0.05 MPa. Three replicates were conducted in above measurement.

2.3. Measurement of NO content

Twenty fruits were cut into small pieces from the pericarp of the tomato equator area at 0 min, 15 min, 30 min, 1 h, 4 h, 8 h, 16 h, 24 h, 48 h (2 d), 96 h (4 d), 144 h (6 d), 192 h (8 d) and 240 h (10 d) and were frozen in liquid nitrogen and stored at −80 °C until used for measurements of NO content. Above samples were used to measure ethylene biosynthesis enzyme activities, expression of related gene about ethylene biosynthesis and expression of related gene about protein phosphorylation.

Extraction and determination of NO content of frozen samples was measured according to the method of Zheng et al. (2014). The content of NO was expressed as μmol per g FW. Three replicates were conducted in above measurement.

2.4. Measurement of ethylene biosynthesis key enzyme activity

Extraction and determination of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) activity were conducted according to the method of Bulens et al. (2011). Enzyme activity was expressed as nmol per g FW per h. Three replicates were conducted in above measurement.

2.5. Expression of related gene about ethylene biosynthesis and protein phosphorylation

Total RNA was isolated using the *EasyPure* Plant RNA Kit (Beijing Transgen Biotech Co. Ltd., Beijing, China) and 1 μg of RNA was used to synthesize the first-strand cDNA with M-MLV Reverse Transcriptase (Beijing Transgen Biotech Co. Ltd., Beijing, China) according to the manual's protocol. Semi-quantitative RT-PCR was performed according to the following description. The changes in expression of *SlACSs*, *SlACOs*, *SlCDPKs* and *SlMAPKs* were studied using TransStart Top qPCR SuperMix (Beijing Transgen Biotech Co. Ltd., Beijing, China). The 100 ng cDNA samples were used as templates and mixed with 500 nM of each primer (Table 1) and 12.5 μL 2 × TransStart Top qPCR SuperMix, and the volume of the reaction system was complemented with 25 μL of free RNA water. PCR reactions were performed using the following parameters: 95 °C, 2 min, followed by (95 °C, 15 s; 60 °C, 20 s; 72 °C, 30 s) followed by 40 cycles in 96-well, optical reaction plates (Bio-rad, Hercules, CA, USA). The SYBR GREEN I fluorescence signal was detected during the annealing step at 60 °C. To check the specificity of each product, melting curve analysis (55–95 °C) was carried out at the end of the amplification protocol. To determine the relative transcript levels among all samples, the threshold cycle (Ct) value was normalized to *SlACTIN* Ct value and calculated using the formula $2^{-\Delta\Delta Ct}$.

Primers for *SlACSs*, *SlACOs*, *SlCDPKs* and *SlMAPKs* were designed from cDNA sequences on deposit in the Nucleotide Database of the National Center for Biotechnology Information using the Primer 6.0 Software (Table 1). The volume of each cDNA sample was