Journal of Plant Physiology 168 (2011) 1820–1827

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Methyl jasmonate-induced defense responses are associated with elevation of 1-aminocyclopropane-1-carboxylate oxidase in *Lycopersicon esculentum* fruit

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ARTICLE INFO

Article history:
Received 7 December 2010
Received in revised form 10 May 2011
Accepted 11 May 2011

Keywords:
ACO
Ethylene
Gene expression
Methyl jasmonate
Tomato fruit

SUMMARY

It has been known that methyl jasmonate (MeJA) interacts with ethylene to elicit resistance. In green mature tomato fruits (*Lycopersicon esculentum* cv. Lichun), 0.02 mM MeJA increased the activity of 1-aminocyclopropane-1-carboxylate oxidase (ACO), and consequently influenced the last step of ethylene biosynthesis. Fruits treated with a combination of 0.02 MeJA and 0.02 α-aminoisobutyric acid (AIB, a competitive inhibitor of ACO) exhibited a lower ethylene production comparing to that by 0.02 mM MeJA alone. The increased activities of defense enzymes and subsequent control of disease incidence caused by *Botrytis cinerea* with 0.2 mM MeJA treatment was impaired by AIB as well. A close relationship ($P < 0.05$) was found between the activity alterations of ACO and that of chitinase (CHI) and β-1,3-glucanase (GLU). In addition, this study further detected the changes of gene expressions and enzyme kinetics of ACO to different concentrations of MeJA. *LeACO1* was found the principal member from the ACO gene family to respond to MeJA. Accumulation of *LeACO1/3/4* transcripts followed the concentration pattern of MeJA treatments, where the largest elevations were reached by 0.2 mM. For kinetic analysis, K_m values of ACO stepped up during the experiment and reached the maximums at 0.2 mM MeJA with ascending concentrations of treatments. V_{max} exhibited a gradual increase from 3 h to 24 h, and the largest induction appeared with 1.0 mM MeJA. The results suggested that ACO is involved in MeJA-induced resistance in tomato, and the concentration influence of MeJA on ACO was attributable to the variation of gene transcripts and enzymatic properties.

Introduction

Ethylene is a classic plant hormone, responsible for the regulation of plant growth and development. It has been proven as a signaling compound in disease resistance during biotic stress (Lin et al., 2009). The biosynthesis of ethylene starts from the conversion of S-adenosyl-l-methionine to 1-aminocyclopropane-l-carboxylic acid (ACC) by ACC synthase (ACS), and is followed by ACC oxidase (ACO)-catalyzed conversion of ACC to ethylene (Yang and Hoffman, 1984). With the characterization of the biosynthetic pathway, the key role of ACS as the rate determining step is widely accepted (Lin et al., 2009). For ACO, the early observations initially suggested that ACO activity was constitutive and did not regulate the biosynthetic pathway (Yang and Hoffman, 1984; Theologis, 1992). However, Alexander and Grierson (2002) listed in their review that a rise in ACO activity precedes ACS activity in preclimacteric fruit in response to ethylene, indicating that ACO activity might involve in the control of ethylene production. In addition, later evidence has shown that ACO expression is tissue-specific and developmentally regulated, supporting a regulatory role of ACO on ethylene production. In tomato, *LeACO1* is predominately expressed in floral tissues, fruit and senescent leaf tissue, *LeACO2* is expressed in the anther, while *LeACO3* can be detected in all of the floral organs excluding the sepals, and is expressed at a low level in senescent leaf tissue (Barry et al., 1996). *LeACO4* is expressed in fruit, and the expression is increased during ripening (Nakatsuka et al., 1998). Examination of ACO mRNA expression patterns in petunia, mungbean and other climacteric fruit such as melon, avocado, apples and bananas were also reported and reviewed by Jiang and Fu (2000). All together, these examples indicate that for these species, at least, the conversion of ACC to ethylene does represent an additional control point in the biosynthesis of ethylene.

The levels of ACO transcripts have also been shown to be regulated by ethylene itself and other phytohormones. Chae et al.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; AIB, α-aminoisobutyric acid; CHI, chitinase; GLU, β-1,3-glucanase; K_m, Michaelis constant; MeJA, methyl jasmonate; PR, pathogenesis-related; qRT-PCR, quantitative real time polymerase chain reaction; V_{max}, maximum velocity.

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doi:10.1016/j.jplph.2011.05.021

(2003) found that *OsACO2* transcripts in etiolated rice (*Oryza sativa L.*) seedlings were increased by indole-3-acetic acid treatment; the ethylene induction of *OsACO3* transcription was blocked by auxin. Gibberellic acid has also been reported to contribute to ACO expression in *Fagus sylvatica* seeds (Calvo et al., 2004); and there has been evidence that ACO can be induced by wounding and pathogens as well (Smith et al., 1986; Díaz et al., 2002).

Methyl jasmonate (MeJA) functions as a mediator of responses to external stimuli, such as wounding, chilling, and pathogen invasion (Rohwer and Erwin, 2008). It has been suggested that MeJA application can influence ethylene production and thus is able to elicit resistance to biotic stress in horticultural products (Xu et al., 1994). Up-regulation of ACO expression was observed by microarray analysis in jasmonates-responsive genes of *Arabidopsis thaliana* (Jung et al., 2007; Matthes et al., 2010). Also in our previous study, we found that MeJA induced lipoxygenase activity and the superoxide radicals formed by lipoxygenase may affect ACO activity (Yu et al., 2009). To clarify this question further, the present study was dedicated to understanding the role of MeJA on ACO gene expressions and enzyme kinetics and its consequences on the elicitation of resistance to the fungal pathogen *Botrytis cinerea* in tomato fruit.

Materials and methods

Fruit materials and treatments

Green mature tomatoes (*Lycopersicon esculentum* cv. Lichun) were hand-picked 48 ± 1 d after flowering from a greenhouse at the Xiaotangshan Geothermal Special Vegetable Base, Beijing. All fruits were selected for uniform shape, color, size and the absence of visible defects. The calyxes of the fruits were removed before treatment.

Tomato fruits were surface-sterilized with 2% (v/v) sodium hypochlorite for 2 min, washed with tap water and air-dried. Fruits were immersed in either (1) water (the control), (2) 0.05, 0.2, 1.0 or 5.0 mM MeJA (MeJA was dissolved in 5 mL 10% ethanol prior dilution in water), (3) 0.2 mM AIB (Aldrich Chemical Company, Milwaukee, WI, USA), an inhibitor of ACO activity, and (4) 0.2 mM AIB + 0.2 mM MeJA solutions in a 50 L stainless steel vacuum container and vacuum infiltrated under low pressure (−35 kPa) for 0.5 min, then kept in the solution under air pressure for 2 min and air-dried afterwards. All fruits were stored at 25 ± 1 °C with 85–90% RH.

Determination of ethylene production and ACC content

Samples were taken at 3, 6, 12 and 24 h after treatments for measurement of ethylene production and ACC content.

Ethylene was assayed by incubating five fruits from each treatment in a 9 L airtight chamber for 1 h at room temperature (25 °C). A 1 mL sample of the headspace gas was withdrawn using a gas-tight syringe and injected into a gas chromatograph (GC-14C, Shimadzu, Japan) equipped with a GDX-502 column and a flame ionization detector. The column temperature was 50 °C and the injection temperature was 120 °C. The carrier gas was N_2 with a rate of 50 mL min^{-1} (McKeon et al., 1982). The results presented are the mean of three replicates.

Pericarp tissue (3 g) from five fruits each time was homogenized using a cold pestle and mortar with 0.5 g polyvinylpyrrolidone in 10 mL 80% ethanol at 4 °C and centrifuged at 10,000 × *g* for 25 min. The supernatant was evaporated under a vacuum at 50 °C. Residues were dissolved in 5 mL distilled water and 2.5 mL chloroform. After storage at 4 °C overnight, 1 mL of the aqueous solution was mixed with 0.1 mL $HgCl_2$ (80 mM) and 2.5 mL distilled water in test-tubes. The tubes were sealed with a stopper and rubber septum and 0.4 mL 5% NaOH–NaClO solution (1:2, v/v) was injected into the tubes, shaken and incubated for 2 min. 1 mL of the gaseous portion was removed and assayed for ethylene as described above. The ACC content was expressed as nmol g^{-1} FW (Lizada and Yang, 1979).

Measurement of disease symptoms

Botrytis cinerea was incubated on potato dextrose agar medium. After incubation at 20 °C for 7 d, spore suspensions of the strain were prepared by flooding the culture dishes with sterile distilled water containing 0.05% Tween-80. The spore suspension was adjusted to 1×10^5 conidia per mL with a hemocytometer. Inoculations were carried out 24 h after fruit were treated with water (the control), 0.2 mM MeJA, 0.2 mM AIB, or 0.2 mM AIB + 0.2 mM MeJA. Ten fruits per treatment were sterilized with 70% ethanol and wounded with a sterile nail at three points (4 mm deep × 2 mm wide) on the equator of each fruit. 10 μL spore suspension was injected into each wound site and the fruit were incubated at 20 ± 1 °C with 90–95% RH. Disease incidence was recorded on 4 d after inoculation and was expressed as the percentage of inoculation spots showing grey mold symptoms.

Determination of defense enzymes activity

Samples were taken at 0, 3, 6, 12 and 24 h after treatments. All enzyme extraction procedures were conducted at 4 °C. Three grams of pericarp tissue from five fruits were homogenized in 5 mL of 0.1 M acetic acid buffer (pH 5.0) for chitinase (CHI, EC 3.2.1.14) and β-1,3-glucanase (GLU, EC 3.2.1.39). The homogenates were centrifuged at 10,000 × *g* for 5 min at 4 °C. The supernatants were used for enzyme assays.

Enzyme activity of CHI was measured according to the method of Boller et al. (1983). CHI activity was expressed as U mg^{-1} protein, where one unit was defined as the formation of 1 nM N-acetyl-D-glucosamine produced per hour. GLU was assayed by measuring the amount of reduced sugar released from the substrate by the dinitrosalicylate method (Wood and Bhat, 1988). GLU activity was expressed as U mg^{-1} protein, where one unit was defined as the formation of 1 μM glucose equivalents per hour. Protein concentrations were determined according to the method of Bradford (1976), using bovine serum albumin as a standard.

Quantitative real-time PCR

Samples of the control and 0.05, 0.2, 1.0 and 5.0 mM MeJA-treated fruits were taken from five fruits at 0, 1, 3, 6, 12, 18 and 24 h after treatment for determination of gene expression. Total RNA was isolated from 3 g pericarp tissue using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). The reverse transcriptions were carried out using 2 μg total RNA treated with RNase-free DNase I and M-MLV reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed with an ABI PRISM® 7000 Sequence Detection System (Perkin-Elmer Applied BioSystems, CA, USA) using the version 1.1 software for data analysis (Brown et al., 2003). The sequences of all primers used are presented in Table 1. For each PCR, 20 ng cDNA were used with SYBR® Green I MasterMix (Toyobo, Osaka, Japan) according to the manufacturer's protocol. All experiments were run in triplicate with different cDNAs synthesized from three biological replicates. Relative quantification of ACO RNA expression was performed using the comparative Ct method. To determine relative fold differences for each sample, the Ct value was normalized to the Ct value for *Ubi3* and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$.