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Vitamin B6 biosynthetic genes expression and antioxidant enzyme properties in tomato against, *Erwinia carotovora* subsp. *carotovora*

Murugesan Chandrasekaran [a,*], Se Chul Chun [b]

[a] Department of Food Science and Biotechnology, Sejong University, 209 Neungdong-ro, Gwangjin-gu, Seoul 05006, Republic of Korea
[b] Department of Bioresource and Food Science, Konkuk University, Gwangjin-gu, Seoul, Republic of Korea

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ABSTRACT

Vitamin B6 (VitB6) is an essential cofactor for >140 biochemical reactions. Also, VitB6 is a potent antioxidant and helps plants cope with both biotic and abiotic stress conditions. However, the role of VitB6 in plant disease resistance has yet to be confirmed using molecular biology approaches. Here, we analyzed the expression patterns of VitB6 biosynthetic genes, including the *de novo* (*PDX1* [*PDX1.2* and *1.3*] and *PDX2*) and the salvage (*SOS4*) pathways during the response to *Erwinia carotovora* subsp. *carotovora*. By quantitative PCR, we found that the most significant upregulation in the transcript profile of *PDX2*, which showed a 9.2-fold increase in expression at 12 h post inoculation (hpi) compared to 24–48 hpi. We also detected significant upregulation of *PDX1.2* and *PDX1.3*, which were 6.6- and 4.3-fold upregulated at 24 hpi compared to 12 hpi, while *SOS4* showed only low-level expression. Also, at 24 hpi, a significant increase in superoxide dismutase, catalase, peroxidase, and polyphenol oxidase activities was observed in plants. Our findings confirm that the expression of *de novo* and *salvage* pathway genes is induced by *E. carotovora* and that this plays an important role in the regulation of defense response by modulating cellular antioxidant capacity.

1. Introduction

A vitamin is defined as an organic compound required in limited amounts by an organism, including human that cannot produce it and thus needs to take it up with the diet. The vitamin B complex comprises water-soluble cofactors and their derivatives that are essential contributors to diverse metabolic processes in plants, animals, and microorganisms [1]. Vitamin B6 (further referred to as VitB6) is a highly versatile cofactor that is required for >140 different biochemical reactions in the cell, primarily associated with amino acid metabolism [2,3]. It is most important for its contribution to amino acid biosynthesis where it serves as a cofactor for enzymes involved in decarboxylation, transamination, deamination, racemization and trans sulfuration [2,4]. VitB6 comprises a group of three chemically related compounds, pyridoxine (PN), pyridoxamine (PM), and pyridoxal (PL), which differ in their 4′-position by having either a hydroxyl, an amino, or an aldehyde group, respectively [2]. To function as active cofactors they need to be phosphorylated at their 5′-position. In plants, two different pathways are known to synthesize VitB6. First, the *de novo* biosynthetic pathway is well resolved and involves the determined activities of the pyridoxine biosynthesis proteins PDX1 and PDX2, that form a multimeric protein complex to synthesize pyridoxal-5′-phosphate (PLP) as an active cofactor [5,6]. Second, the *salvage* pathway is a recovery pathway that converts PN, PL, or PM to the active cofactor PLP by the concerted activities of two enzymes, pdxH and pdxK, a VitB6 oxidase and a VitB6 kinase, respectively. PL is converted to PLP by a PN/PL/PM kinase (PDXK), while synthesis of PLP from PN and PM requires the additional activity of a PNP/PMP oxidase PDX3 [2,7].

Recently, *PDX1* and *PDX2* homologs from plants have been isolated and characterized [8,9,10]. Despite this evidence, the effect of VitB6 on plant development, the mechanisms controlling its biosynthesis and stress tolerance are still poorly understood. In addition, VitB6 is a potent antioxidant and helps plants cope with both biotic and abiotic stress conditions [3,7,8,and 11]. In *A. thaliana*, mutations at *AtPDX1* genes led to increased sensitivity to salt, osmotic, and high light stress [8,12,13] whereas overexpression of *PDX* gene resulted in increased tolerance to oxidative stress [10,14]. During these abiotic stress responses, it was shown that VitB6 vitamers are efficient quenchers of both singlet oxygen and superoxide anions [9,15].

Moreover, Denslow et al. [9] and Sivasubramanian et al. [16] showed that salicylic acid, methyl jasmonate, and ethylene, chemical inducers of oxidative plant defense responses, increase *PDX* transcript in *N. tabacum* and *Hevea brasiliensis*. Expression of the *PDX* genes was induced in tobacco plants after treatment with signaling molecules but was decreased after inoculation with an incompatible pathogen, *Pseudomonas syringae* pv. *phaseolicola* [9]. Also, pyridoxine delayed and attenuated

* Corresponding author at: Department of Food Science and Biotechnology, Sejong University, Seoul, Republic of Korea.
E-mail address: chandrubdubio@gmail.com (M. Chandrasekaran).

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active oxygen-dependent plant defense responses to a bacterial pathogen [14] and reduced singlet oxygen-induced death in *A. thaliana flu* mutant protoplasts [12]. Moreover, *Arabidopsis pdx1.3* mutant plants showed an elevated expression level for the pathogenesis-related proteins (PR-5) gene [17]. These findings imply that the VitB6 vitamers might play important roles in regulating plant defense responses against different types of pathogens.

While plants produce reactive oxygen species (ROS) as by-products of normal processes including photosynthesis, photorespiration, and metabolism, during times of stress the levels of ROS often reach damaging levels [18–19]. To counteract the detrimental effects associated with increased ROS during stress, cells employ a host of compounds and enzymes including antioxidant enzymes such as superoxide dismutases (SODs), catalases (CATs), polyphenol oxidases (PPOs) and peroxidases (PODs) [18–21]. In addition to the identification of the novel pathway for VitB6 biosynthesis, recent information has also uncovered that VitB6 vitamers, similar to vitamins C and E, show antioxidant activity and may be an important component of cellular antioxidant defenses [4]. Denslow et al. [11] showed that B6 vitamers are efficient quenchers of both singlet oxygen and superoxide and have antioxidant activity. However, direct molecular evidence supporting the hypothesis that VitB6 as an antioxidant function in plant defense is still lacking. Therefore, the objective of this study was to investigate the impact of *Erwinia carotovora* subsp. *carotovora* inoculation on VitB6 biosynthetic genes expression and defense-related enzyme responses in tomato.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. carotovora subsp. *carotovora* YKB133061 was obtained from Korean Type Culture Collection, Republic of Korea and was maintained on LB agar medium. The bacteria were maintained in tryptic soy broth (TSB) supplemented with 20% glycerol at −80 °C for long-term storage. For experimental use, the bacterial strain was transferred to tryptic soy agar (TSA) and incubated at 30 °C for 24 h. The bacterial cells were harvested by centrifugation at 10,000*g* for 10 min. The cell precipitate was resuspended in 10 mM $MgCl_2$ and the cell concentration of bacterial suspensions of *E. carotovora* was adjusted to 10^8 colony-forming unit (CFU)/mL ($OD_{600} = 1.0$).

2.2. Plant materials and growth conditions

Tomato seeds were obtained from Korea seed resource center. Seeds were surface sterilized with 70% ethanol for 5 min, followed by three washing with water. Four-week-old tomato seedlings were transplanted into plastic pots containing fertilized bed soil. For gene expression and biochemical analyzes, plants were subjected to the following treatments: (i) mock control plants treated with 4% maltose and 1% peptone solution (ii) pathogen inoculation with *E. carotovora*. Plants were grown in mixed soil (soil: sand: vermiculite, 4:1:1 v/v) in a growth chamber at 23 ± 2 °C and 60% relative humidity. Three treatments with three replicates per treatment were maintained in a completely randomized block design.

2.3. Real-time PCR analysis of VitB6 biosynthetic gene expression in response to E. carotovora inoculation

2.3.1. Total RNA isolation and cDNA synthesis

Total RNA was extracted from 100 mg of frozen tomato tissue using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. RNA samples were treated with DNAse to remove the genomic DNA contamination. The integrity of total RNA was verified by formaldehyde agarose gel electrophoresis. The purity and concentration of the total RNA were determined spectrophotometrically (NanoDrop ND-1000 Spectrophotometer; Celbio, Italy). RNA was reverse transcribed to cDNA using the First-strand cDNA synthesis kit (Bioneer, Korea).

2.3.2. Quantitative PCR (qPCR) assays

The sequences of primers used for RT-PCR analysis were as shown in Table 1. Primer specificity and amplification efficiency were also verified for each gene with melting curve analysis (after 40 cycles) and agarose gel electrophoresis. The qPCR reaction was performed in a 25 μL reaction mixture containing 5 μL of diluted cDNA sample, 12.5 μL of AccuPower® 2× Greenstar qPCR Master Mix (Bioneer, Korea), and 0.4 μM of each forward and reverse primer (Table 1). The qPCR reactions were carried out in the CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA) using the following PCR cycling conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 20s and annealing and extension at 60 °C for 45 s. The tomato actin gene was used as the internal control for normalization of the expression of the target genes. The fold change in the expression level of the VitB6 gene transcripts expression levels was calculated using CFX Manager Software (Bio-Rad). The qPCR experiments were carried out with three independent total RNA samples.

2.4. Antioxidant enzymes

2.4.1. SOD activity

We used the SOD activity kit (Enzo Life Sciences Inc., USA) for SOD activity (Catalog # ADI-900-157). We transferred 25 μL of the 1× SOD buffer to the activity control wells. We added 25 μL of sample to the appropriate wells and then mixed with 150 μl of Master Mix into each well. Their action was initiated by adding 25 μL of 1× xanthine solution to all the wells using multichannel pipet. The total SOD activity was estimated using a microtitre plate reader, at 450 nm.

2.4.2. CAT activity

The catalase fluorometric detection kit (Enzo-Catalog # ADI-907-027) is used to detect CAT activity by measuring the amount of H_2O_2 remaining after sample addition according to the manufacturer's protocol. The reaction mixture contained 50 μL of enzyme extract and 50 μL of 40 μM H_2O_2. They were incubated at room temperature for 60 min. After

Table 1
List of primers used in the study.

Gene name	Host plants	Primer name	Primer sequence (5′ → 3′)
Actin	*S. lycopersicum*	*SlActin1-F*	AGG CAC ACA GGT GTT ATG GT
		SlActin1-R	AGC AAC TCG AAG CTC ATT GT
SOS4	*S. lycopersicum*	*SlSOS4-rt-1F*	GCG TAT TTC ACG GGA ACT GG
		SlSOS4-rt-1R	CTT GAG ACA GCA AGC TCT GC
PDX2	*S. lycopersicum*	*SlPDX2-rt-1F*	CAA AGC TTC GGA ACG CGT TCA A
		SlPDX2-rt-1R	GTC AAT GAG TAG CCA TTT GAC C
PDX1.2	*S. lycopersicum*	*SlPDX1.2-rt-1F*	GAT GCA GCT GGG TTG TGA TG
		SlPDX1.2-rt-1R	TCC AAA CCA CTG CTA GCC GC
PDX1.3	*S. lycopersicum*	*SlPDX1.3-rt-1F*	CAT GTG CGT TCC GTT ATG GG
		SlPDX1.3-rt-1F	TGA ACC ACA GGG AGC CTA CC