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刘长命 等/甜瓜 S-腺苷甲硫氨酸脱羧酶基因的克隆及白粉病诱导表达分析

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·农业生物技术·

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摘　要：为探讨 *S*-腺苷甲硫氨酸脱羧酶 (*S*-adenosylmethionine decarboxylase，SAMDC) 基因在甜瓜抵御白粉病菌中的作用，根据已知 EST 序列和甜瓜基因组数据库，在甜瓜抗白粉病品种'Yuntian930'中克隆获得该基因的全长编码序列，命名为 *CmSAMDC* (GenBank 登录号为 KF151861)。生物信息学分析表明，*CmSAMDC* 的主开放阅读框 (mORF)长 1 095 bp，编码 364 个氨基酸，预测分子量为 40 kDa。聚类分析表明，CmSAMDC 预测蛋白与黄瓜和四季橘中该蛋白的同源关系最近，并与其他双子叶植物聚为一类。原核表达分析表明，CmSAMDC 以融合蛋白形式表达，相对分子量约为 40 kDa，与预测一致。实时定量表达分析表明，*CmSAMDC* 基因受白粉病诱导表达，在接种后 48 h 表达量达到峰值，为接种前的 7 倍，并且在甜瓜的根、茎、叶、卷须中均有表达。结果提示该基因可能参与了甜瓜的抗白粉病反应。

：甜瓜，白粉病，S-腺苷蛋氨酸脱羧酶，基因克隆，表达分析

Cloning and analysis of a powdery mildew responsive gene *CmSAMDC* from *Cucumis melo* L.

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Abstract:　In order to investigate the effect of *S*-adenosylmethionine decarboxylase (SAMDC) gene in melon resistance to powdery mildew, according to the previous results of EST sequences from cDNA-AFLP library and the melon genome sequence data, the *SAMDC* gene was isolated from Chinese wild melon clone 'Yuntian930' by RT-PCR and designated as

CmSAMDC (GenBank Accession No. KF151861). The mORF (main open reading frame) was 1 095 bp encoding 364 amino acids with a molecular mass of 40 kDa. The predicted protein sequence showed high similarity with *Cucumis sativus* and *Citrofortunella microcarpa*. The SDS-PAGE showed that the expression protein was a fusion protein with the molecular weight of 40 kDa, which perfectly matched the mass calculated from the amino acid sequence. Quantitative real-time PCR analysis indicated that the expression level of *CmSAMDC* reached a maximum at 48 hpi (hours post inoculation) that over 7-fold to 0 hpi, and the expression of *CmSAMDC* was also detected in tendril, root, leaf and stem. These results indicate that *CmSAMDC* gene may play protective roles in melon resistance to powdery mildew infection.

Keywords: *Cucumis melo*, powdery mildew, SAMDC, gene clone, expression analysis

Muskmelon is one of the most important cultivated plants worldwide. However, in China, melon was threatened by various abiotic and biotic stresses[1]. Powdery mildew, which is strictly biotrophic and completely dependent on the living host cells for survival[2], is a common disease of melon under field and greenhouse conditions worldwide. Many recent studies have shown that *Podosphaera xanthii* (*P. xanthii*) is predominant in most countries[3-5]. Based on our previous study[6], it could be preliminarily confirmed that the *P. xanthii* race 2F is the predominant physiological race within cucurbitaceous plants in Guanzhong areas, Shaanxi Province of China.

Currently, control of powdery mildew on melon is mainly achieved by widespread application of various fungicides. Although powdery mildew can be controlled by fungicides, the cost and the environmental impact of residues are undesirable consequences, and the long term use has led to fungicide resistance of powdery mildew races[7]. Furthermore, with the accumulation of toxic heavy metals in soil, the exceeded level of relevant metal ions will be found in melon, which seriously affects the melon food safety. Compared with the application of fungicides, using resistant cultivars is a more effective and environmentally safe approach to disease control.

Plants use various mechanisms to defend against pathogens, and these are often initiated by plant disease resistance genes, which may encode cell receptors that detect the presence of a specific pathogen and these initiate the activation of signal transduction pathways. Polyamines (PAs) are small ubiquitous compounds that have been implicated in the regulation of many physiological progresses and a variety of stress responses in plants[8]. The enhanced level of polyamines plays an important role in the protective response of plants to various abiotic stresses[9]. SAMDC is one of the key regulatory enzymes in the biosynthesis of polyamines. There are some reports about *SAMDC* genes responses to abiotic stresses in plants[10]. However, very few study about biotic stresses was conducted in melon.

In our previous study using cDNA-AFLP method, we observed an EST (expression sequence tag) that was induced in leaves of 'Yuantian930' upon infection with *P. xanthii*[6]. We analyzed the EST sequence and found that it was the only one completely consistent with *SAMDC* in melon genome sequence database (https://melonomics.net/). Since 'Yuantian930' has been shown to be highly resistant to powdery mildew, we undertook the present study to gain a better understanding of SAMDC in melon and verified its potential role in powdery mildew resistance. We cloned the full-length of *CmSAMDC* gene from 'Yuantian930' and further analyzed its expression in response to *P. xanthii*. The aim of this study was to determine whether or not there was a relationship between melon resistance and the response of *CmSAMDC* to *P. xanthii* infection. To our knowledge, this is the first report of *SAMDC* gene potentially related to powdery mildew resistance in melon.

1　Materials and methods

1.1　Plant material and inoculation method

The powdery mildew (PM) resistant clone, Chinese wild melon *C. melo* 'Yuantian930' was used in this study, which was provided by the melon and watermelon research group of Horticulture

Institute of Northwest A&F University, China. The *P. xanthii* isolate used in this work was collected from natural infections in cucurbit and through mono bacterial colony propagation. The melon seedlings were grown in the plant growth chamber at 30 ℃/18 ℃ (day/night) operating a 16 h/8 h (light/dark) photoperiod and 70%–85% relative humidity. PM inoculation were performed by spraying plants with a suspension of 10^6 sporangia/mL *P. xanthii* conidiophores, and then the plants were kept in the same conditions. The leaves from the infected plants (at the forth-leaf stage) were collected after 0 h, 12 h, 24 h, 48 h, 72 h and 120 h, stored at –80 ℃. As a control, leaves were collected from plants treated with sterile water only.

1.2　RNA extraction and cDNA synthesis

Total RNA was extracted from melon leaves using the Trizol reagent following the instructions of the Trizol kit (Tiangen, Beijing). Residual DNA was removed by DNase Ⅰ (Promega, Madison, WI, USA). Concentration of total RNA was measured with an ultraviolet spectrophotometer (V-550, JASCO, Japan) at 260 nm. RNA purity was checked by determining the A_{260}/A_{280} ratio, and RNA integrity was examined by 1% agarose gel electrophoresis. Smart^{TM} RACE cDNA Amplification Kit was used to synthesize cDNA, following the instructions of the Clontech kit (Clontech-TaKaRa Bio Inc., Japan).

1.3　Cloning of melon *CmSAMDC*

On the basis of the EST fragment of *CmSAMDC* from Chinese wild melon *C. melo* 'Yuntian-930' by cDNA-AFLP analysis and the data from melon genome sequence database, the gene-specific primers (Table 1) *CmSAMDC*s and *CmSAMDC*a containing full ORF (open reading frame) were designed using Primer Premier 5.0, and the *Bam*H I and *Eco*R I restriction sites were added. PCR amplification of *CmSAMDC* with one microliter of cDNA from infected leaves was added to a PCR mix containing 2 U of *Taq* polymerase (MBI Fermentas), 1× PCR buffer, 2.5 mmol/L MgCl$_2$, 0.1 mmol/L of each dNTP, and 0.5 mmol/L of the forward and reverse degenerate primers. The

final volume of each reaction was 25 μL and PCR was performed using a Bio-Rad thermocycler following conditions: initial denaturation at 94 ℃ for 3 min; followed by 35 cycles of 94 ℃ for 30 s, 56 ℃ for 1 min, 72 ℃ for 1 min; and a final extension at 72 ℃ for 10 min. The PCR products were electrophoresed in 1.5% agarose gel. The fragments of expected size was isolated and purified using the Gel Extraction Kit (BioTake, USA). The pGEM-T Easy Vector System (Promega, Madison, USA) was used for DNA cloning. The positive clones identified by PCR and enzyme digestion were sequenced by the Beijing Aoke Biotech Co. Ltd. (Beijing, China).

1.4　Sequence analysis

The similarity analysis of nucleotide and protein sequences was carried out using the Blast tool at NCBI (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequence was analyzed with the Predict Protein Analysis System (http://www.predictprotein.org/). The parameters for the given protein were computed using the ProtParam tool at the ExPASy Proteomics Server (http://web.expasy.org/protparam/). The transmembrane regions and orientation of given protein was predicted using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The multiple sequences alignment was performed with Clustal X and Genedoc software, and a phylogenetic tree was constructed using the MEGA 5.1 software by the Neighbor-joining method. The reliability of each node on phylogenetic tree was tested by bootstrapping 1 000 times.

表 1　所用引物序列
Table 1　Primers used in this study

Primer name	Primer sequence (5′−3′)
*CmSAMDC*s	GC<u>GGATCC</u>ATGACGTGTCCAACCTCTG
*CmSAMDC*a	C<u>GAATTC</u>CTAATACTTCCCAACTTCCTCG
*CmSAMDC*f	ATCAAAACTTGCGGCACTAC
*CmSAMDC*r	AGCACCCTCACAATCAACTTAG
*Actin*S	TGCCCAGAAGTTCTATTCCAGC
*Actin*A	CATAGTTGAACCACCACTGAGGAC

The cutting sites of *Bam*H Ⅰ and *Eco*R Ⅰ were underlined.

1.5　Quantitative real-time PCR and data analysis

The first-strand cDNA was synthesized using the Reverse Transcription System Kit (Promega, USA) according to the manufacturer's instructions. A pair of gene-specific primers (Table 1) *CmSAMDC*f and *CmSAMDC*f were designed following the recommended guidelines for qRT-PCR primer design, and primers specific for the melon *Actin* gene (GenBank Accession No. AY859055) were used as the internal control (Table 1). The product size was 147 bp.

Relative levels of gene expression were analyzed with the Themal Cycler Dice® Real Time System (Bio-Rad, Hercules, CA, USA) and normalized with the results for *Actin*. Each 20 μL PCR reaction contained 10 μL of SYBR® *Premix ExTaq*™ Ⅱ (2×), 0.8 μL of PCR Forward Primer (10 μmol/L), 0.8 μL of PCR Reverse Primer (10 μmol/L), 2 μL of cDNA (200 ng/μL), and 6.4 μL of ddH$_2$O. The PCR cycling conditions consisted of an initial polymerase activation step at 95 ℃ for 30 s, followed by 40 cycles of 95 ℃ for 5 s and 60 ℃ for 30 s. Output data generated by the instrument on-board software iQ5 (Bio-Rad) were transferred to Sigmaplot software (v.10.0, Systat Inc., CA, USA) for analysis. Real-time quantitative RT-PCR was performed in three replicates for each sample. In addition, reverse transcription negative control was included to check for potential genomic DNA contamination. The relative expression of *CmSAMDC* gene was calculated according to the method of $2^{-\triangle\triangle Ct}$. The $\triangle\triangle CT=(C_{T,Target}-C_{T,Actin})_{Timex}-(C_{T,Target}-C_{T,Actin})_{Time0}$, $C_{T,Target}$ means the C_T value of target gene, $C_{T,Actin}$ means the C_T value of control gene, Time x means the post inoculation time (12 h, 24 h, 48 h, 72 h, 120 h), Time 0 means the time before inoculation (0 h).

1.6　Expression of *CmSAMDC* in *E. coli*

After verifying the sequence, the recombinant plasmid of positive clone was digested with *Bam*H I/*Eco*R I and ligated directly into the prokaryotic expression vector pET-28a (Fermentas, USA), which was predigested with the same enzymes. A recombination reaction yielding a pET-28a and *CmSAMDC* fusion construct was conducted. After sequencing and confirming by restriction digestion, the positive recombinant prokaryotic expression plasmids, designated pET28a-*CmSAMDC*, were then transformed into competent *E. coli* strain BL21 (DE3) for expression of the fusion protein. Transformed *E. coli* cells were incubated at 37 ℃ overnight in Luria-Bertani medium with 50 mg/L Kan. 1 mL of the overnight cultures were added into 50 mL of fresh LB medium containing Kan and incubated with shaking at 37 ℃ until OD_{600} reached 0.6. Isopropyl-β-d-thiogalactoside (IPTG) was added to a final concentration of 1 mmol/L and the incubation was continued at 37 ℃. 2 mL of thallus were collected at 2 h, 4 h, 6 h and 8 h respectively after IPTG was added, followed by centrifuging at 12 000×g for 30 s, and then resuspended with 5× buffer solution [every 10 mL reaction contained: 0.6 mL Tris-HCl (1 mol/L, pH 6.8), 5 mL glycerol (50%), 2 mL SDS (10%), 0.5 mL mercaptoethanol, 1 mL bromophenol blue (1%), 0.9 mL ddH$_2$O]. The resulting lysate was heated at 100 ℃ for 10 min, and then centrifuged at 12 000×g for 1 min. The supernatant was used as a sample for SDS-PAGE. After electrophoresis, the gel was stained with Coomassie Brilliant Blue.

2　Results and discussion

2.1　Isolation of *CmSAMDC* gene

Based on the study conducted before, a length of 479 bp product was found highly identical to *SAMDC* in *Cucumis sativus*, *Brassica juncea* and *Arabidopsis thaliana*[11]. Given this, the full ORF of *SAMDC* gene with a length of 1 095 bp was amplified from 'Yuntian 930' and named *CmSAMDC* (GenBank Accession No. KF151861). Results from agarose gel electrophoresis showed that the PCR products had the expected molecular size of 1 095 bp, which suggested that the full length of *CmSAMDC* gene was successfully amplified by gene special primers (Fig. 1).
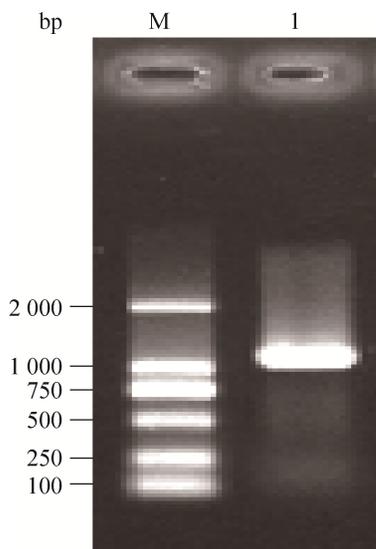
图 1 *CmSAMDC* 基因的扩增

Fig. 1   Amplification of *CmSAMDC* gene. M: DL2000 marker; 1: the *CmSAMDC* cDNA.

2.2　Bioinformatics analysis of *CmSAMDC*

The results showed that the deduced peptide consists of 364 amino acid residues, with a predicted molecular weight of 40 kDa, and theoretical pI of 4.61. The transmembrane regions and orientation was predicted that the given protein probably has no transmembrane region (Fig. 2). Phylogenetic analysis comprising the SAMDC protein sequences from twenty plants revealed that the *CmSAMDC* protein had close distance with *Cucumis sativus*, *Arabidopsis thaliana*, *Brassica juncea* and *Citrofortunella microcarpa*, as they all belong to dicotyledon, and the lowest similarity was detected as to *Saccharum officinarum*, *Triticum aestivum* and *Zea mays*, since the last three belong to monocot (Fig. 3).

In this study, the expected size of *SAMDC* gene in melon was isolated, and the sequence analyses indicated that the *CmSAMDC* from 'Yuantian930' shares a high level of sequence homology with other plants of *SAMDC* genes [12-15]. Phylogenetic analysis and the alignment of the deduced amino acid sequences with other plants revealed a series of common features, which was consistent with former studies[16].
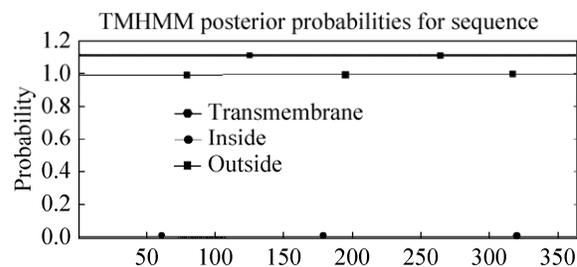
图 2　跨膜域与方向的预测

Fig. 2   The transmembrane regions and orientation prediction. The line with solid dot and solid square means the polypeptide inside or outside the membrane respectively; the line with solid hexagon means the transmembrane region.

2.3　Expression of *CmSAMDC* in *E. coli* BL21 (DE3)

After the array and orientation of the insert sequence were confirmed by restriction digestion with enzyme *Eco*R I /*Bam*H I and sequencing, the PCR product was directly cloned into pET-28a. Sequencing results showed that *CmSAMDC* gene has been inserted correctly into pET-28a vector (Fig. 4). Upon IPTG induction, fusion proteins containing the desired protein were expressed in *E. coli* BL21 (DE3). As illustrated by the SDS-PAGE (Fig. 5), the fusion protein was detected as a broad and a predominant band at 43 kDa (including 3 kDa His-tag) in the induced cell lysate with pET28a-SAMDC (lane 4 h and 6 h), and six hours of IPTG induction would be suitable. The molecular mass was consistent with the molecular weight calculated from the amino acid sequence. No reaction was observed with no-loading vector and 0 h as well.

2.4　Expression analysis of *CmSAMDC*

The expression level of *CmSAMDC* in response to *P. xanthii* in PM-resistant clone 'Yuntian930' was investigated at different time by qRT-PCR. As shown in Fig. 6, *CmSAMDC* was constitutively expressed at low levels in leaves of 'Yuantian930' before inoculation, then it was peaked at 48 h as almost 7 times high as observed in control. These results suggested that transcription of *CmSAMDC* is indeed upregulated in Chinese wild PM-resistant melon *C. melo* 'Yuntian-930' upon infection of *P. xanthii* pathogens.
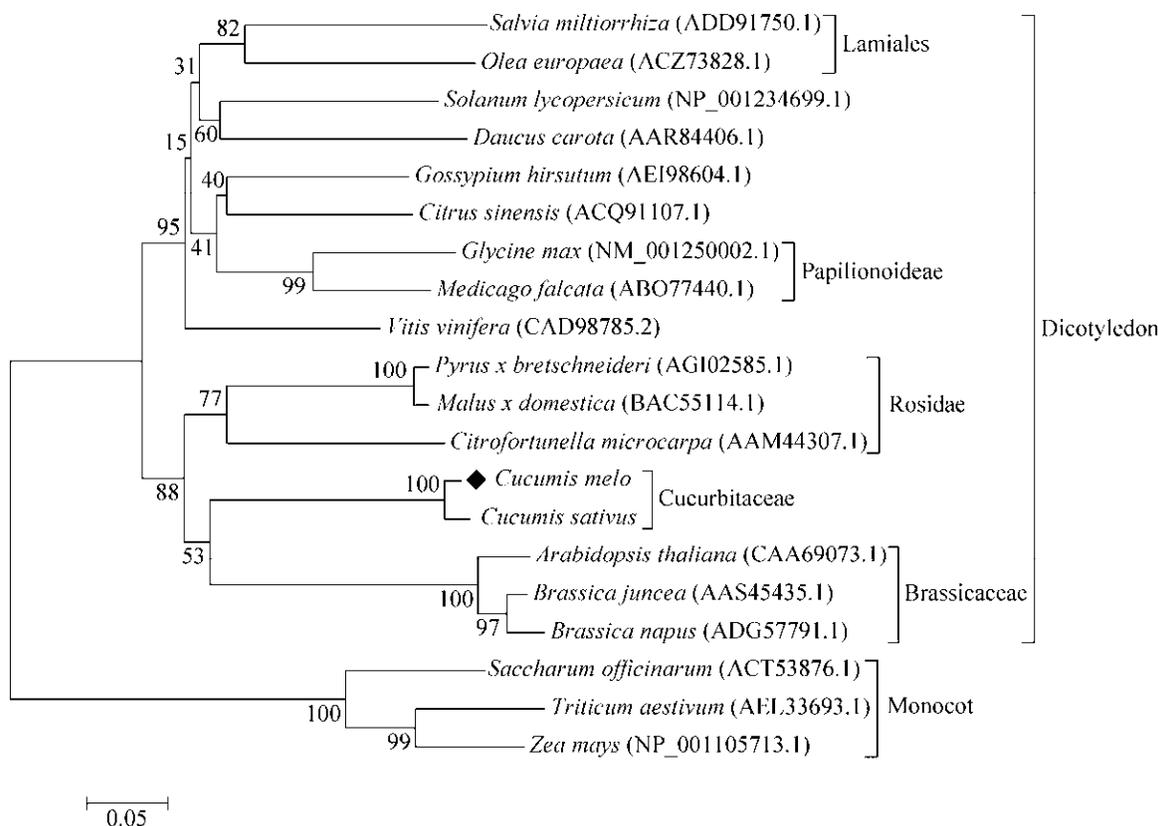
图 3　20 种植物 SAMDC 蛋白氨基酸进化树分析

Fig. 3　Phylogenetic tree analysis based on the alignment of deduced amino acid sequences of SAMDC protein in 20 plants. Two major classes were identified and classified to dicotyledon and monocot. The numbers below the branches refer to the percentage of 1 000 bootstrap replications supporting the nodes.
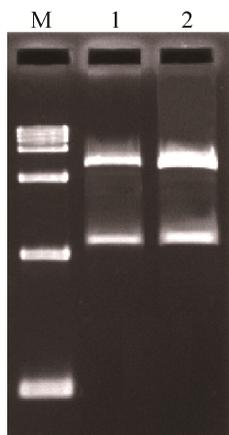

图 4　融合表达载体的酶切鉴定

Fig. 4　Validation of fusion expression vector by digestion. M: DNA marker (250, 1 000, 2 500, 5 000, 10 000, 12 500, 15 000 bp); 1: pGEM-T-SAMDC digested with *Eco*R Ⅰ and *Bam*H Ⅰ; 2: pET28a-SAMDC digested with *Eco*R Ⅰ and *Bam*H Ⅰ.
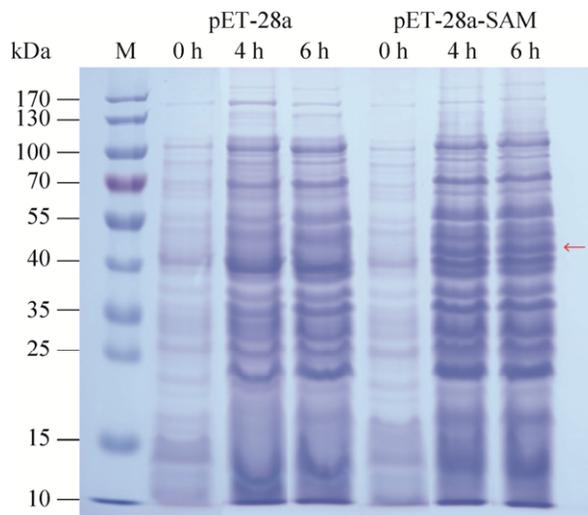

图 5　pET-28a-*CmSAMDC* 重组体的原核表达分析

Fig. 5　*CmSAMDC* proteins expressed in *E. coli* and separated by SDS-PAGE. The arrows showed the molecular weight of the target protein agreed well with the predicted.
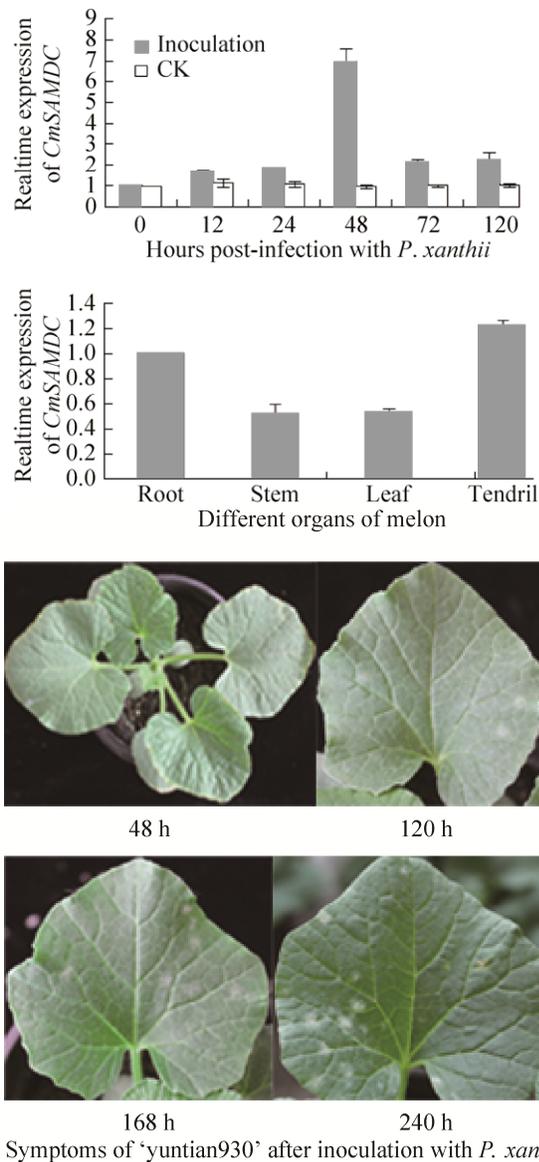
48 h   120 h

168 h   240 h

Symptoms of 'yuntian930' after inoculation with *P. xanthii*

图 6 中国野生甜瓜白粉病菌侵染后 *CmSAMDC* 基因表达分析

Fig. 6 Expression profiles of *CmSAMDC* gene in Chinese wild melon after *P. xanthii* infection. Each experiment was repeated three times, and the standard error bars were indicated. Total RNA was extracted from leaves of *C. melo* clone 'Yuntian930', and analyzed at 0 h, 12 h, 24 h, 48 h, 72 h and 120 h after inoculation with *P. xanthii*. The leaves of the plants on which sterile water was sprayed were collected as the control leaves (CK).

For coping with biotic and abiotic stresses, plants have evolved a wide range of defense mechanisms. These defense mechanisms depend on a series of proteins, including many involved in regulating cellular response to stress and signaling crosstalk[17]. Many of these proteins involved the production of phytohormones, pathogenesis-related proteins and transcription factors[18]. The pathogen defense mechanism in plants has been reported to be associated with early and strong gene expression[19]. Most plant resistance genes are transcriptionally regulated in response to pathogen attack. In rice, the transcription of resistance gene *Xa1* appears to increase following pathogen inoculation[20], which indicates that the transcription of the resistance gene depends on the type of plant-pathogen interaction.

During this experiment, we have examined the expression pattern of *SAMDC* in PM-resistant clone 'Yuantian930' in response to *P. xanthii* and it showed higher levels and stronger expression, which was almost 7 times as high as observed in no pathogen inoculation. This trend is similar to *SAMDC* expressing in sugarcane[13], tall fescue[14] and tomato under abiotic stress[21]. In addition, the author's further research also shows that *SAMDC* gene plays protective roles in melon resistance to powdery mildew infection by transgenic arabidopsis plants expressing *CmSAMDC*[22]. Thus, *SAMDC* was involved in *P. xanthii*-induced defense response besides the important role in response to abiotic stress[23-26]. However, the close relationship of SAMDC and powdery mildew stress need to be further verified.

3　Conclusion

In summary, we have demonstrated that *CmSAMDC* gene was induced by *P. xanthii* in PM-resistant melon leaves and likely plays a positive role in defense mechanisms that confer resistance to powdery mildew in melon. Further investigations of *CmSAMDC* may aid us in understanding the mechanism(s) of PM resistance in melon.

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