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刺葡萄*VdMAPK7*参与炭疽病胁迫响应的功能分析

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摘 要:【目的】克隆刺葡萄*VdMAPK7*基因,并对其参与炭疽病胁迫响应的功能进行分析。**【方法】**结合前期转录组数据,以刺葡萄福安(*Vitis davidii* 'Fu'an')为试材,通过实时荧光定量PCR(quantitative real time PCR,qRT-PCR)分析*VdMAPK7*基因在炭疽菌侵染后不同时间点表达水平变化,通过聚类分析VdMAPK7蛋白与其他物种相关MAPK蛋白的系统发育关系。利用烟草叶片亚细胞定位技术分析VdMAPK7蛋白在细胞中的位置。在番茄中异源表达*VdMAPK7*基因后,番茄果实接种尖孢炭疽菌(*Colletotrichum acutatum*),验证其对炭疽病胁迫的响应。**【结果】**刺葡萄*VdMAPK7*基因响应炭疽菌诱导后表达量逐渐升高,在接种第7天达到高峰。VdMAPK7蛋白与欧洲葡萄、番茄、马铃薯、茶树、珙桐、烟草聚为一大类;亚细胞定位发现,VdMAPK7蛋白定位在细胞质和细胞核中;*VdMAPK7*转基因番茄植株矮于野生型植株,果实变小。转基因番茄果实接种尖孢炭疽菌后,相较于野生型番茄,*VdMAPK7*基因过表达番茄果实发病较轻;qRT-PCR结果显示,*VdMAPK7*基因过表达番茄植株中响应水杨酸信号通路的基因*SlPR1*和*SlPR2*上调表达。**【结论】***VdMAPK7*基因在番茄中过量表达均可增强对尖孢炭疽菌的抗性,推测*VdMAPK7*基因参与了葡萄对炭疽菌的胁迫响应。

关键词:刺葡萄;炭疽病;MAPK基因

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Functional analysis of *VdMAPK7* from Chinese wild spine grape (*Vitis davidii* Föex) in response to *Colletotrichum viniferum*

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Abstract:【Objective】Mitogen activated protein kinase (MAPK) cascade pathway, a major signal transduction pathway widely distributed in eukaryotes, has an important function in plant responses to various biotic and abiotic stresses. Transcriptome analysis of Chinese wild spine grape (*Vitis davidii* Föex) infected by *Colletotrichum viniferum* was performed in a previous study. The infected grape fruits at 7 dpi showed significant changes in gene expression, and that the up-regulated genes were enriched for those MAPK cascade, calcium ion binding and serine/threonine kinase. The *VdMAPK7*, selected from transcriptome data, showed highly up-regulated expression during *C. viniferum* infection, indicating that the *VdMAPK7* was responsible for the resistance to *C. viniferum*. The main objective of this study was to clone and verify the function of the *VdMAPK7* in response to *C. viniferum*.【Methods】Chinese wild spine grape (*V. davidii* 'Fu'an') was used as the experimental materials and the *VdMAPK7* was selected based on the transcriptome data for further study. The grape berries were inoculated by *C. viniferum* (strain FJ017) then the exocarps were collected after inoculation at different infection time points (0 d, 1 d, 3 d and 7 d) for further analysis. The RNA from *C. viniferum* infected grape exocarp were extracted by lithium chloride precipitation method and reverse transcription and cDNA

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synthesis were performed using PrimeScript™ RT kit with gDNA Eraser. The expression patterns of the *VdMAPK7* at different time points after *C. viniferum* infection were analyzed by qRT-PCR using *GAPDH* (CB973647) as reference gene with $2^{-\Delta\Delta Ct}$ method. CDS sequence of the *VdMAPK7* were cloned and the amino acid sequence of the VdMAPK7 was compared based on NCBI database, then other related proteins with high homology were screened. The phylogenetic relationship between the VdMAPK7 protein and other related proteins were analyzed by cluster analysis using Phylogeny.fr platform. The full-length CDS sequence of the *VdMAPK7* was obtained by sequencing, and the amino acid sequence of the VdMAPK7 was compared based on NCBI database. Sequence multiple alignment was carried out using DNAMAN (v6.0) and demonstrated with GenDoc v2.7.0. The *VdMAPK7* was cloned into pCambia2300 vector and introduced into *Agrobacterium tumefaciens* GV3101, then the VdMAPK7 was transiently expressed in the tobacco leaves for subcellular localization and finally observed in confocal laser endomicroscopy (Leica TCS SP8) with excitation at 488 nm to elucidate the specific location in the cells. To study the response of the *VdMAPK7* against *C. viniferum*, transgenic tomato plants of the *VdMAPK7* were obtained using leaf disc transformation and inoculation assay of *C. acutatum* in the transgenic tomato of *VdMAPK7* were carried out. The transgenic tomatoes inoculated by *C. acutatum* were collected after inoculation at different infection time points (0 h, 6 h, 12 h, 24 h, 48 h, 72 h), RNA extraction and cDNA synthesis were performed as described above. The expression patterns of the resistance genes (*SlPR1* and *SlPR2*) were analyzed by qRT-PCR using *SlActin* as reference gene.【Results】Chromosomal location of the *VdMAPK7* analysis was performed using the Blat- Search option. *VdMAPK7* could be mapped on chromosome 4 of the European grape reference genome, which was distributed in the region 18 972 351−18 978 329 bp. The amino acid homology identity of the VdMAPK7 cloned in this study compared with the VvMAPK7 (*V. vinifera*) was 99.18%. The results showed that the VdMAPK7 and the MAPKs from other species all contained S_TKc domain (32−319 aa). The phylogenetic tree analysis showed that the VdMAPK7 was clustered with homologous proteins from *V. vinifera*, *Solanum lycopersicum*, *S. tuberosum*, *Camellia sinensis*, *Davidia involucrata* and *Nicotiana tabacum*. The results from qRT-PCR showed that the *VdMAPK7* could response to the induction of *C. viniferum*, and highly expressed at the 7 days after inoculation. The results indicated that the *VdMAPK7* could be induced to express in response to *C. viniferum*. The activated *Agrobacterium* solution containing the *VdMAPK7* was transiently expressed into the *N. benthamiana* leaves and the fluorescence distribution was observed under a laser confocal microscope. The subcellular localization assay showed that the *VdMAPK7* was localized in cytoplasm and nucleus. The transgenic tomato plants of the *VdMAPK7* were obtained by leaf disk transformation method, and PCR detection was carried out using gDNA from transgenic plant leaves. Then, RNA from candidate plants (#2, #3, #9, #14, #21) was extracted and reverse transcribed, specific primers were designed according to the sequence of the *VdMAPK7*, and semi-quantitative PCR detection was carried out with *SlActin* as an internal reference, #2 and #9 plants were finally identified and selected for further study. Compared with the wild-type tomatoes, the *VdMAPK7*-overexpressing plants were shorter, with curled leaves and smaller fruits. To further verify the difference between the wild-type tomatoes and the *VdMAPK7*-overexpressed tomatoes, the single fruit weight, transverse diameter and longitudinal diameter of tomato fruit were measured in the study. The *VdMAPK7* transgenic tomato fruits and the wild-type tomato fruits were inoculated with *C. acutatum* and the results showed that the over-expressed *VdMAPK7* lines enhanced the resistance to *C. acutatum*. The results of qRT-PCR showed that *SlPR1* and *SlPR2* in response to salicylic acid signaling pathway were up-regulated in the *VdMAPK7* over expressed tomato fruits inoculated with *C. acutatum*. The re-

sults showed that the *VdMAPK7* could enhance the resistance to *C. acutatum* in the transgenic tomatoes. 【Conclusion】The *VdMAPK7* could enhance the resistance of grape to *C. viniferum* and these results would provide theoretic basis for molecular breeding of spine grape for the resistance to *C. viniferum*.

Key words: *Vitis davidii* Föex; *Colletotrichum viniferum*; MAPK gene

促分裂原活化蛋白激酶（mitogen activated protein kinase, MAPK）级联途径是真核生物中广泛存在的信号转导途径，并在植物应对各种生物和非生物胁迫、激素以及在细胞分裂和发育过程中起关键作用[1-5]。MAPK信号通路通常包括3个功能相互关联的蛋白激酶：促分裂原活化蛋白激酶激酶激酶（MAPKKK/MAP3K/MEKK）、促分裂原活化蛋白激酶激酶（MAPKK/MAP2K/MKK）和促分裂原活化蛋白激酶（MAPK/MPK）。MAPKKK通过信号分子受体或者自身感知外界刺激而被磷酸化激活，MAPKKK活化后，通过磷酸化下游MAPKK中的保守结构（S/T-X3-5-S/T）来激活MAPKK。MAPKK是一类双重特异性激酶，能磷酸化下游的MAPK的保守氨基酸基序T-X-Y（TDY或TEY）中的苏氨酸（T）和酪氨酸（Y），使MAPK激活[1]。磷酸化的MAPKs作用于细胞质或细胞核中不同的蛋白，重新编码基因表达，使植物表现出抗逆性[6]。

植物MAPK级联在植物抵御病原菌攻击的信号转导中起着重要作用，MAPK级联参与多种防御反应的信号传导，包括植物胁迫/防御激素的生物合成/信号转导、活性氧（reactive oxygen species，ROS）的产生、防御基因激活以及植物生理功能生物合成[7]。已有研究对拟南芥[1, 8]、水稻[9]、杨树[10]、玉米[11]、苹果[12]中MAPK级联各家族基因组成进行了分析。在紫花苜蓿、烟草中也发现了参与病原菌或病毒胁迫的MAPK级联途径[13-14]。

芪合成酶（stilbene synthase，STS）是葡萄中与抗病相关的白藜芦醇合成过程最后一步的催化酶，研究表明芪类化合物合成需要MAPK级联反应，特异性MAPK级联抑制剂（PD98059）可以有效抑制STS的激活[15]。王刚[16]对葡萄中的MAPK类激酶家族做了鉴定分析，结果表明葡萄中有45个MAPKKK激酶、5个MAPKK激酶和12个MAPK激酶。葡萄中MAPKKKs激酶可以响应生物、非生物胁迫和植物激素的处理；葡萄中*VvMKK3*基因可以响应白粉菌的诱导上调表达；葡萄的12个MAPKs中，部分MAPKs参与了葡萄组织的生长发育，在葡萄白粉菌侵染下，*VvMPK1*、*VvMPK9*和*VvMPK10*基因上调表达。毛葡萄丹凤-2中，*VqMAPKKK38*基因可以响应葡萄白粉菌、盐害和冷害，这是由于*VqMAPKKK38*基因受到过氧化氢和钙信号刺激而被激活，介导了芪类化合物的生物合成，最终参与了ROS和钙信号的调控途径[17]。燕山葡萄中的2个*MAPK*基因和*VyMAPK3*基因可以响应水杨酸（salicylic acid，SA）、茉莉酸（Jasmonic acid，JA）和脱落酸（abscisic acid，ABA）等植物生长调节剂而诱导表达，*VyMAPK2*基因变化不明显。*VyMAPK3*基因可以响应干旱、盐害和低温诱导表达，而*VyMAPK2*基因仅响应干旱诱导[18]。但目前未见葡萄中*MAPK*类基因与葡萄炭疽病之间关系的报道。

炭疽病是影响南方葡萄产业的重要病害，中国葡萄属植物中蕴含丰富的抗炭疽病种质资源，刺葡萄中各株系对炭疽病的抗性极强[19]。笔者在前期炭疽菌侵染下刺葡萄转录组测序的基础上，获得了上调表达的*VdMAPK7*基因，并同源克隆了*VdMAPK7*基因，通过过表达载体构建、番茄遗传转化、表型抗性评价以及亚细胞定位分析，探讨刺葡萄*VdMAPK7*基因参与炭疽病菌胁迫响应的功能，研究其参与炭疽病胁迫响应的抗病机制，对定向改良葡萄抗病性具有重要意义。

1 材料和方法

1.1 供试材料

以福建省福安市穆云乡溪塔村刺葡萄沟中生长良好的刺葡萄福安（*Vitis davidii* ‘Fu’an’）为材料。从9株葡萄中采集健康成熟、大小一致的葡萄果实，设置3次生物学重复（每3株为1次生物学重复，每株14粒果实）。将果实采回，用70%（φ）乙醇表面消毒1 min，前期研究中的葡萄炭疽菌菌株（*Colletotrichum viniferum* FJ017）作为病原菌，通过针刺法接种果粒[20]。接种后，收集不同侵染时间点（0、1、3和7 d）的果皮用于进一步分析，受侵染果皮贮存于−80 ℃冰箱备用。番茄尖胞炭疽菌（*C. acutatum*）由笔者实验室保存，25 ℃培养在马铃薯葡萄糖琼脂培养基

（potato dextrose agar medium，PDA）上，每14 d继代培养1次。商品化番茄（Micro-Tom）种子由笔者实验室保存。

1.2　RNA提取、反转录和*VdMAPK7*基因表达模式分析

采用氯化锂沉淀法提取炭疽菌侵染的葡萄果皮RNA[21]，RNA样品（2 μg）经无RNase的DNA酶Ⅰ处理去除残余的基因组DNA，然后用带有去除gDNA的Primescript™RT试剂盒进行cDNA合成（TaKaRa）。反应混合物用蒸馏水稀释1∶40，作为实时荧光定量PCR（quantitative real time PCR，qRT-PCR）模板。在Bio-Rad iQ5系统（Bio-Rad，USA）中进行定量分析，每个cDNA样本3次重复，并用$2^{-\Delta\Delta Ct}$方法计算相对于葡萄*GAPDH*（CB973647）的转录水平[22]。所有数据均表示为平均标准差（n=3）。根据*VdMAPK7*基因的序列设计定量引物：qPCR-MAPK7-F：GGGAACCTCCTTGTGAATGC；qPCR-MAPK7-R：AGCGGGTGACAACATACTCA，进行qRT-PCR并对*VdMAPK7*基因的表达模式进行分析。

1.3　*VdMAPK7*基因克隆

根据转录组获得*VdMAPK7*基因序列，与葡萄基因组网站和GenBank中的序列进行比对，最终确认*VdMAPK7*基因序列。并根据*VdMAPK7*基因序列（蛋白质编码区序列，CDS）和pCambia2300的载体序列选择合适的酶切位点，最终设计扩增引物（p2300-VdMAPK7-*BamH*Ⅰ-F：tcggtacccggggatccATGGCCACTCGAGTTGAGCCTCC；p2300-VdMAPK7-*Sal*Ⅰ-R：gctcaccatggtgtcgacGGCATGAGAACAAACAGCTTCAGGA），用高保真酶KOD-Plus-Neo（Toyobo，北京）进行PCR扩增，反应体系和程序均按照说明书步骤进行。参照博迈德公司（Biomed，北京）的无缝克隆试剂盒进行同源重组反应，将上述获得的胶回收产物与线性化载体pCambia2300进行重组反应后转化至大肠杆菌感受态，挑取单克隆测序鉴定，最终获得正确的重组质粒。

1.4　*VdMAPK7*生物信息学分析

将VdMAPK7氨基酸序列放到BLAST网站上进行序列比对，并将不同物种的同源序列下载，用于构建系统进化树。使用DNAMAN（v6.0）进行多重比较以确定VdMAPK7的氨基酸序列相似性。使用GenDoc（v2.7.0）软件输出序列多重比较分析结果。*VdMAPK7*基因在染色体上位置预测在葡萄基因组网站Genoscope Genome Browser中分析。系统进化树的构建在Phylogeny.fr platform（http://www.phylogeny.fr）上，采用最大似然法（Maximum likelihood）进行。

1.5　烟草表皮亚细胞定位与转基因番茄鉴定

将测序正确的重组质粒使用电激法转入农杆菌GV3101中，参照Xie等[23]的方法，配制农杆菌重悬液，用无针头的1 mL注射器在健康的本氏烟草叶背面注射入重悬菌液，在光照培养箱中培养72 h，激光共聚焦显微镜下（莱卡TCS SP8）观察烟草叶片中GFP融合蛋白的分布，并保存图片。

采用农杆菌介导的叶盘法[23]转化番茄（Micro-Tom），将0.5 cm×0.5 cm的番茄无菌叶片放入重悬好的农杆菌菌液（OD_{600}=0.5）中悬浮5 min，吸去多余菌液后暗培养2 d，利用卡那霉素进行抗性筛选，42 d后，将获得的抗性芽切下进行生根培养，将生根良好的番茄植株移栽至营养钵中，放入人工气候室中培养，最终获得T_0代转基因番茄植株。

参照Xie等[23]的方法提取番茄叶片DNA进行PCR反应，使用引物（p2300-F：TCCTTCGCAAGACCCTTCCTCTAT；p2300-R：CAGGGTCAGCTTGCCGTAG）检测*VdMAPK7*基因是否整合到番茄基因组中，同时以野生型植株DNA作为阴性对照进行PCR反应，以p2300-VdMAPK7质粒DNA作为阳性对照进行PCR反应。取PCR反应产物进行1%（w）琼脂糖凝胶电泳检测。

为检测*VdMAPK7*基因在转基因番茄中是否表达，选取PCR检测阳性植株的幼嫩叶片，提取总RNA，经过反转录后得到cDNA，利用*SlActin*（SlActinF：ATTCCCTGACTGTTTGCTAGT；SlActinR：TCCAACACAATACCGGTGGT）内参引物和1.2中的*VdMAPK7*基因定量引物进行28个循环的PCR反应，将产物进行1%（w）琼脂糖凝胶电泳半定量检测。

1.6　转基因番茄抗炭疽病鉴定

选取成熟、无病斑的T_0代转基因番茄果实，使用70%（φ）乙醇溶液表面消毒15 s，用无菌水清洗3遍，解剖针在果实顶部造伤，使用6 mm番茄尖胞炭疽病菌PDA菌块（预培养7 d）接种到造伤部位。在28 ℃培养箱进行保湿暗培养，48 h后移出菌块，于接种后的0、6、12、24、48、72 h采集果实果皮，液氮速冻，−80 ℃冰箱保存备用。参照1.2提取

上述样品RNA并反转录为cDNA（100 ng·μL^{-1}），以1.5中番茄*SlActin*为内参基因，采用qRT-PCR的方法，比较番茄在接种炭疽菌后，番茄*SlPR1*（EU589238，SlPR1- F：ATAAAGTGATCGATTGTCGAGGA；SlPR1- R：TAAGCTGCAACATACACACATCC）和*SlPR2*（EU589238，SlPR2-F：TCTGTAGACATGACGTTGATTGG；SlPR2- R：AGAGCATACGGAAGTGAAATCTG）在不同时间段的表达量。

1.7 数据统计与分析

使用SPSS 22.0软件分析数据，利用单因素方差分析和独立样本*T*检验法进行显著性分析（*p<0.05），并用Sigmaplot 12.5作图。

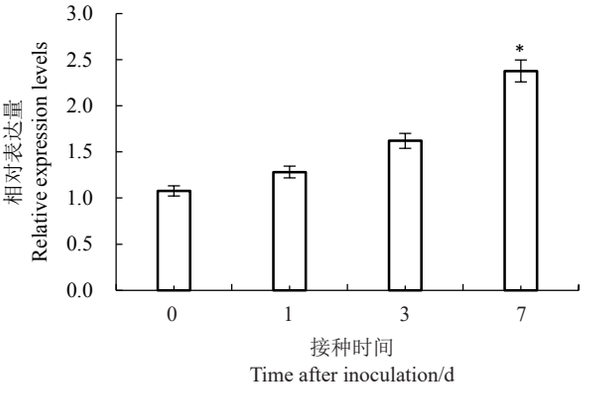
2 结果与分析

2.1 *VdMAPK7*基因的表达模式分析

对刺葡萄福安果实接种葡萄炭疽病菌，接种0、1、3、7 d后果皮中*VdMAPK7*基因表达情况见图1。*VdMAPK7*基因的表达量随着侵染时间延长持续升高，第7天达到高峰。结果表明，*VdMAPK7*基因可以响应葡萄炭疽病而诱导表达。

2.2 *VdMAPK7*基因克隆及其蛋白结构分析

将*VdMAPK7*基因克隆测序的结果在NCBI网站上进行Blastn分析，并将克隆出的*VdMAPK7*（序列号：VIT_04s0023g02420）基因放入葡萄基因组Grape Genome Browser（http://www.genoscope.cns.fr/externe/Genome Browser/Vitis/）中进行比对。应用该网站的Blat-Search选项，对获得的*VdMAPK7*基因进行染色体定位。结果如图2-A所示，*VdMAPK7*基因可以映射（mapping）至欧洲葡萄参考基因组的4号染色体上，分布于18 972 351~18 978 329 bp的区域。



误差线代表SE，3次生物学重复（* p<0.05）。

Each value represents the means ± SE of three different experiments (* p<0.05).

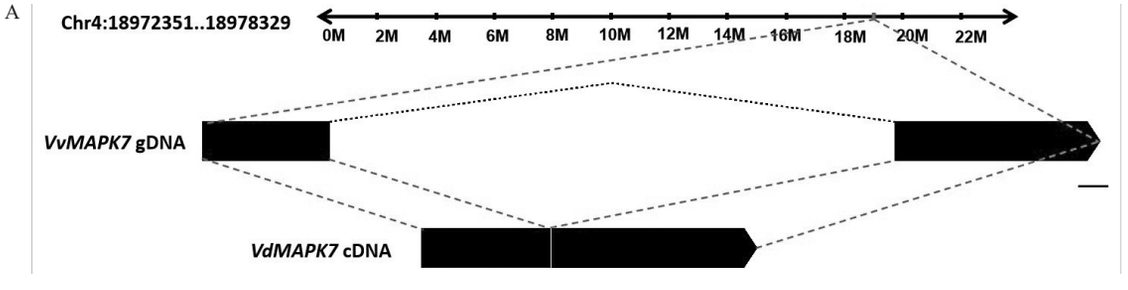
图1 刺葡萄福安*VdMAPK7*基因响应葡萄炭疽病诱导表达

Fig. 1 The qRT-PCR expression analysis of *VdMAPK7* in *Vitis davidii* Föex under *C. viniferum* inoculation

将本研究克隆到的刺葡萄VdMAPK7氨基酸序列与欧洲葡萄MAPK7氨基酸序列进行比对，发现二者氨基酸同源率为99.18%。将VdMAPK7氨基酸序列应用Clustw程序与欧洲葡萄、拟南芥、栽培稻、烟草和番茄进行了比对，结果显示VdMAPK7蛋白与其他物种均含有S_TKc结构域（32~319 aa）（图2-B）。

2.3 VdMAPK7蛋白进化树分析

将刺葡萄中的VdMAPK7氨基酸序列与Gen-

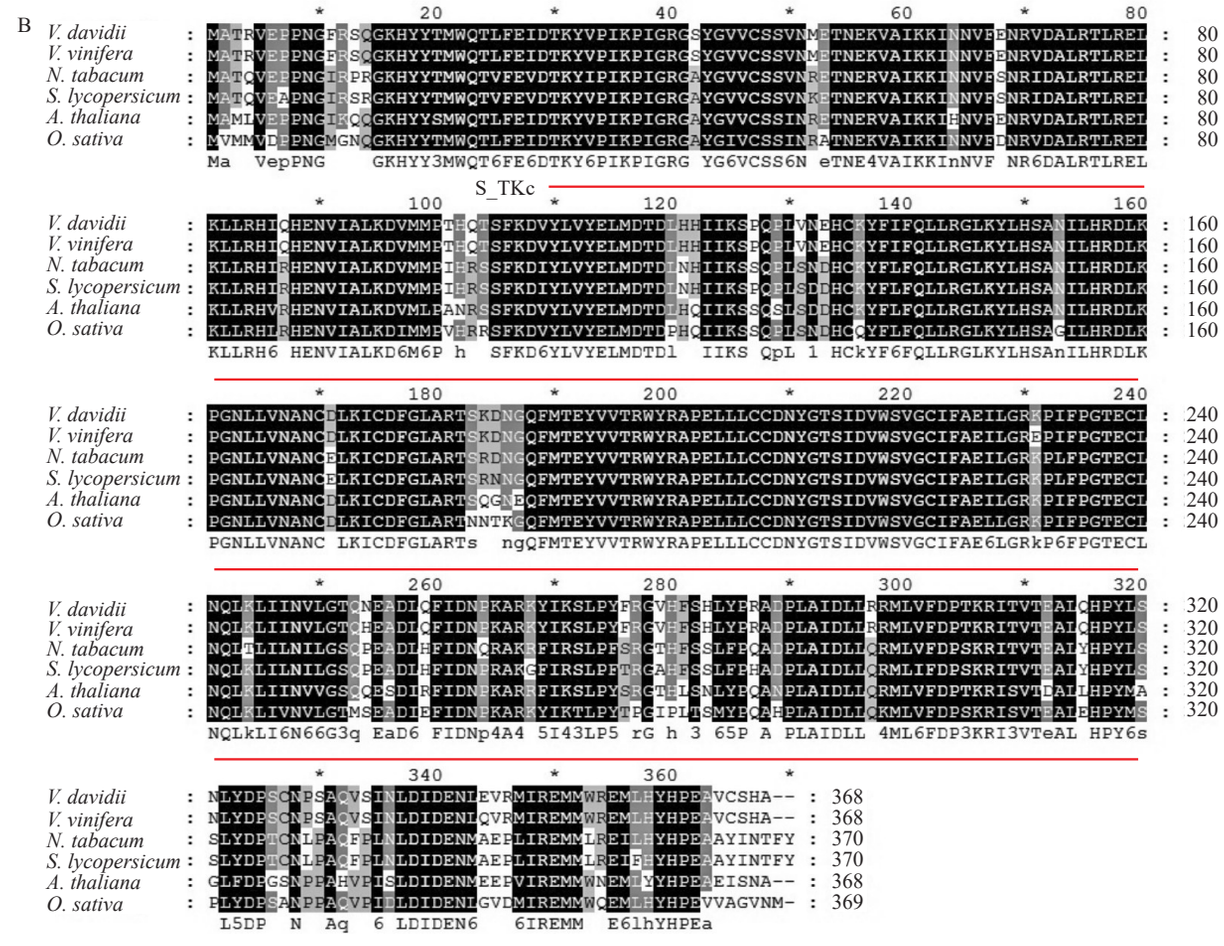


A. 刺葡萄*VdMAPK7*基因染色体定位，Chr4. 4号染色体；标尺．100 bp；gDNA. 基因组DNA；cDNA. 互补DNA。B. 刺葡萄VdMAPK7蛋白结构域分析，刺葡萄（*Vitis davidii* Föex）VdMAPK7与欧洲葡萄（*Vitis vinifera*）、拟南芥（*Arabidopsis thaliana*）、栽培稻（*Oryza sativa*）、烟草（*Nicotiana tabacum*）和番茄（*Solanum lycopersicum*）中的同源蛋白结构域分析。红色横线代表S_TKc结构域。

A. Chromosomal location schematic diagrams of *VdMAPK7* from *Vitis davidii* Föex, Chr4. No.4 chromosome; Scale bar. 100 bp; gDNA. Genomic DNA; cDNA. Complementary DNA. B. Protein domain analysis of VdMAPK7 from *Vitis davidii* Föex, VdMAPK7 from *Vitis davidii* Föex compared with its homologous proteins from *Vitis vinifera*, *Arabidopsis thaliana*, *Oryza sativa*, *Nicotiana tabacum* and *Solanum lycopersicum*. Red line represents S_TKc domain.

图2 刺葡萄*VdMAPK7*基因克隆及其蛋白结构域分析

Fig. 2 Analysis of cloning *VdMAPK7* and protein domain of *VdMAPK7* from *Vitis davidii* Föex



续图 **Continued Figure**

Bank 中下载的其他植物 MAPK 氨基酸序列进行同源性比对，应用 Phylogeny.fr platform 中的最大似然法构建进化树，如图 3 所示。所有进行比对的序列可以分为 3 大类，刺葡萄中的 *VdMAPK7* 基因与欧洲葡萄、番茄、马铃薯、茶树、珙桐、烟草同源基因聚为一大类，且与欧洲葡萄关系最近。

2.4　VdMAPK7 蛋白亚细胞定位分析

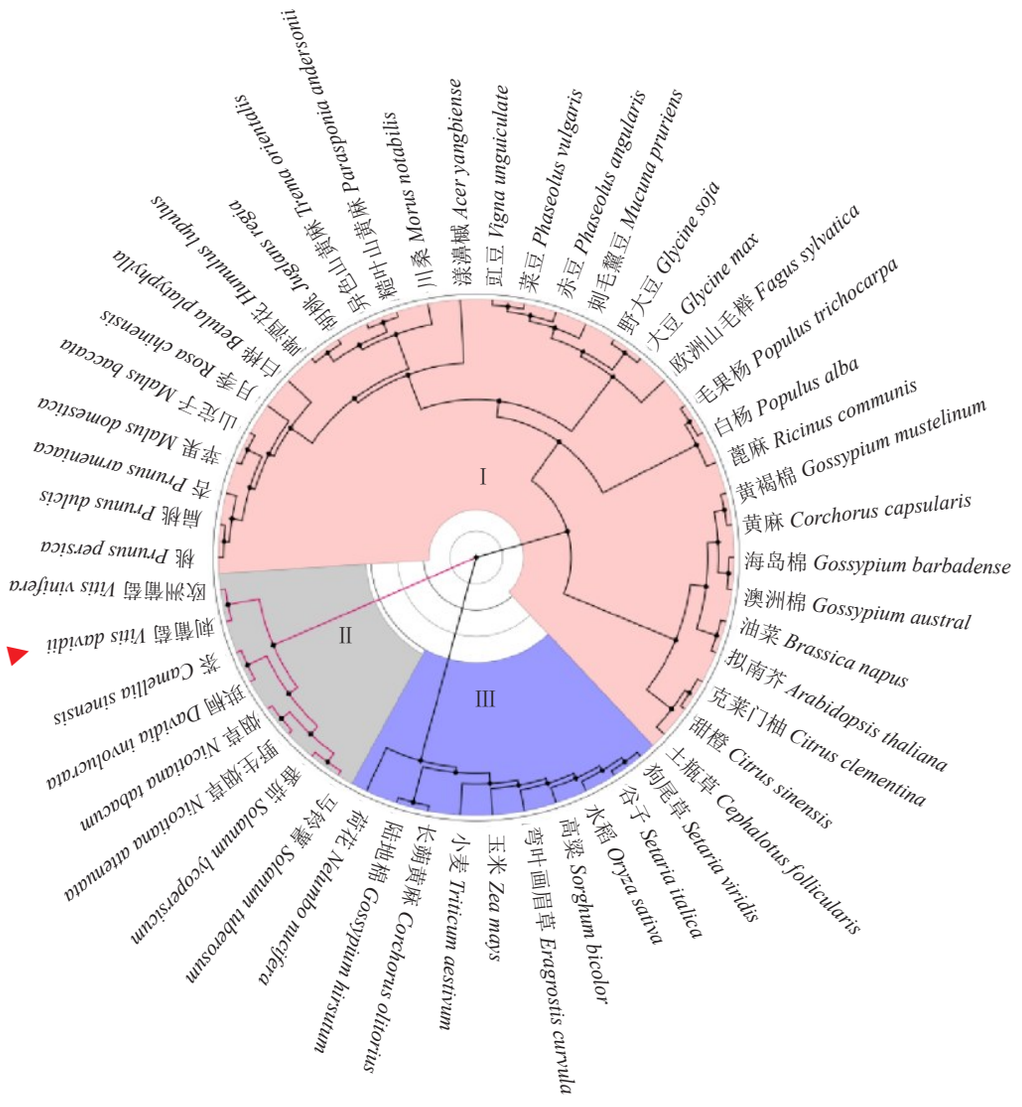
以本氏烟草叶片为材料，将活化后含有 VdMAPK7 蛋白的农杆菌菌液注射到本氏烟草叶片中，培养 3 d 后，在激光共聚焦显微镜下观察荧光分布情况。以细胞核染料 DAPI 作为核定位对照，以 35s-GFP 作为空载体对照。结果如图 4 所示，35s-GFP 定位于细胞质和细胞核上，VdMAPK7-GFP 定位情况与对照一致，同样定位在细胞核与细胞质上。

2.5　*VdMAPK7* 基因过表达番茄植株获得与表型

采用叶盘转化法获得了 *VdMAPK7* 基因过表达番茄植株，将移栽成活的番茄炼苗后，取幼嫩叶片提取 DNA 进行 PCR 检测。以 pCambia2300- VdMAPK7-GFP 质粒为阳性对照，以野生型番茄植株叶片 DNA 为阴性对照，进行 PCR 电泳，结果如图 5-A 所示。候选植株（#2、#3、#9、#14、#21）与阳性对照有一致条带，野生型植株中检测不到电泳条带，说明候选植株可能为转基因植株。为进一步验证 *VdMAPK7* 基因在上述候选植株中是否表达，提取候选植株幼嫩叶片 RNA 并进行反转录，根据 *VdMAPK7* 基因序列设计特异引物，并以番茄 *SlActin* 为内参基因进行 PCR 半定量检测，经过 28 个循环的 PCR 检测，#2 和#9 植株中检测到目的条带，而野生型植株中没有条带，可以断定#2 和#9 植株为阳性植株（图 5-B），*VdMAPK7* 基因在阳性植株（#2、#9）中表达。相较于野生型番茄，*VdMAPK7* 基因过表达植株矮小，叶片卷曲，果实变小（图 5-C~F）。

2.6　*VdMAPK7* 基因过表达番茄果实接种炭疽菌试验

为研究 *VdMAPK7* 基因与抗炭疽病之间的关系，对 *VdMAPK7* 转基因番茄果实和野生型番茄果

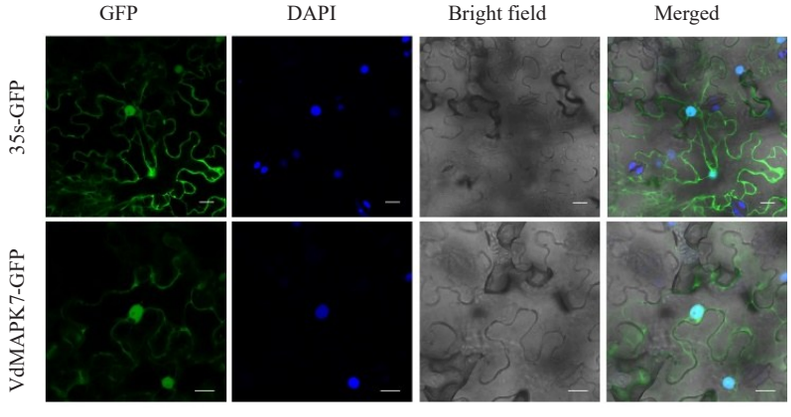


红色箭头代表刺葡萄 VdMAPK7，Ⅰ、Ⅱ和Ⅲ代表不同类别。

Red arrow represents VdMAPK7 from *Vitis davidii* Föex, Ⅰ, Ⅱ and Ⅲ represent different classes.

图 3 刺葡萄 VdMAPK7 蛋白与其他植物 MAPK 同源蛋白序列聚类分析

Fig. 3 Phylogenetic tree of VdMAPK7 from *Vitis davidii* Föex and other homologous protein sequences

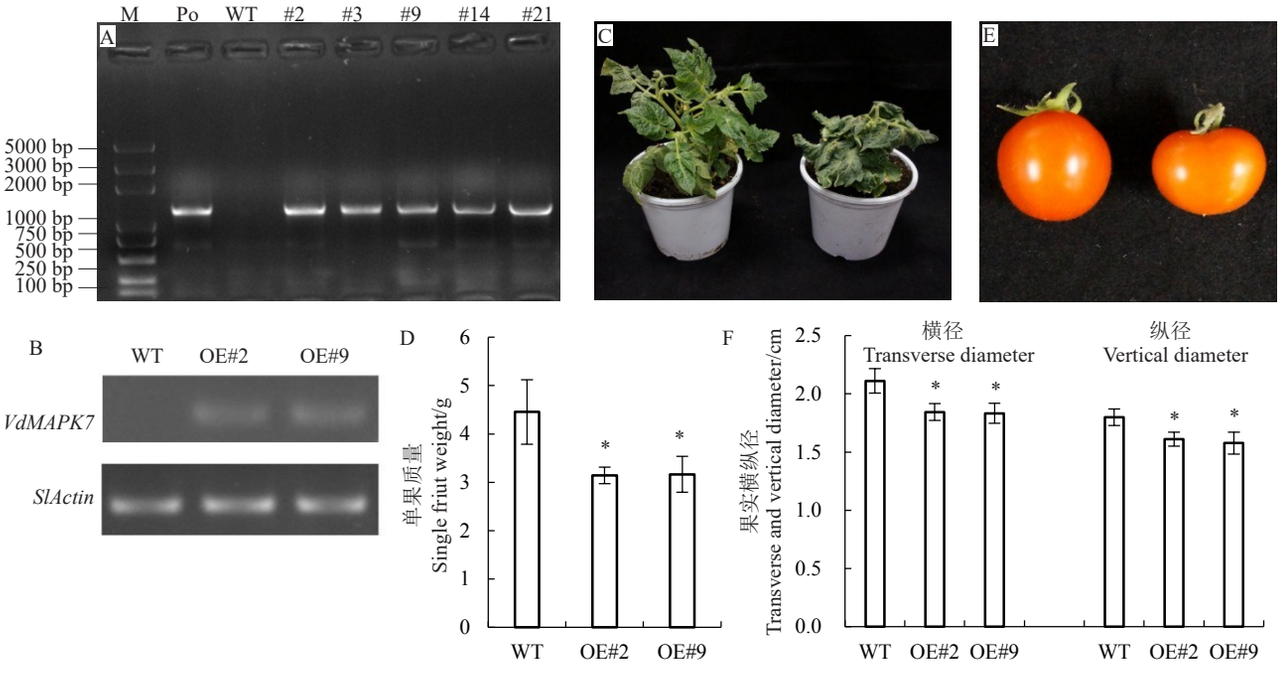


激光通道分别为 GFP 激发荧光下、DAPI 染色荧光、明场图和合并图。35s-GFP，VdMAPK7-GFP 的标尺为 20 μm。

From left to right: GFP; Fluorescent dyes of DAPI; bright field and merged. Scale bars=20 μm.

图 4 VdMAPK7 亚细胞定位分析

Fig. 4 Subcellular localization of VdMAPK7-GFP



A. PCR 检测转基因番茄；B. qRT-PCR 检测转基因番茄。C. 番茄植株（左为野生型，右为转基因型）；D. 单果质量比较；E. 番茄果实（左为野生型，右为转基因型）；F. 果实横、纵径比较。M. Marker；Po. 阳性对照；WT. 野生型。#2、#3、#9、#14、#21. 转基因候选植株；OE. 过表达。误差线代表 *SD*，3 次生物学重复（*p＜0.05）。

A. PCR analysis of *VdMAPK7* from transgenic tomato plants; B. qRT-PCR analysis of *VdMAPK7* from transgenic tomato plants. C. Wild type plant (left) and transgenic plant (right); D. Comparison single fruit weight between wild type tomato and *VdMAPK7* overexpression tomato; E. Wild type fruit (left) and transgenic fruit (right). F. Comparison transverse and vertical diameter between wild type tomato and *VdMAPK7* overexpression tomato. M. Marker; Po. Positive control; WT. Wild type. #2, #3, #9, #14, #21. Transgenic candidate plants; OE. Overexpression. Data are means ± SD based on three independent replicates (*p＜0.05).

图 5　*VdMAPK7* 过表达的转基因番茄鉴定与表型

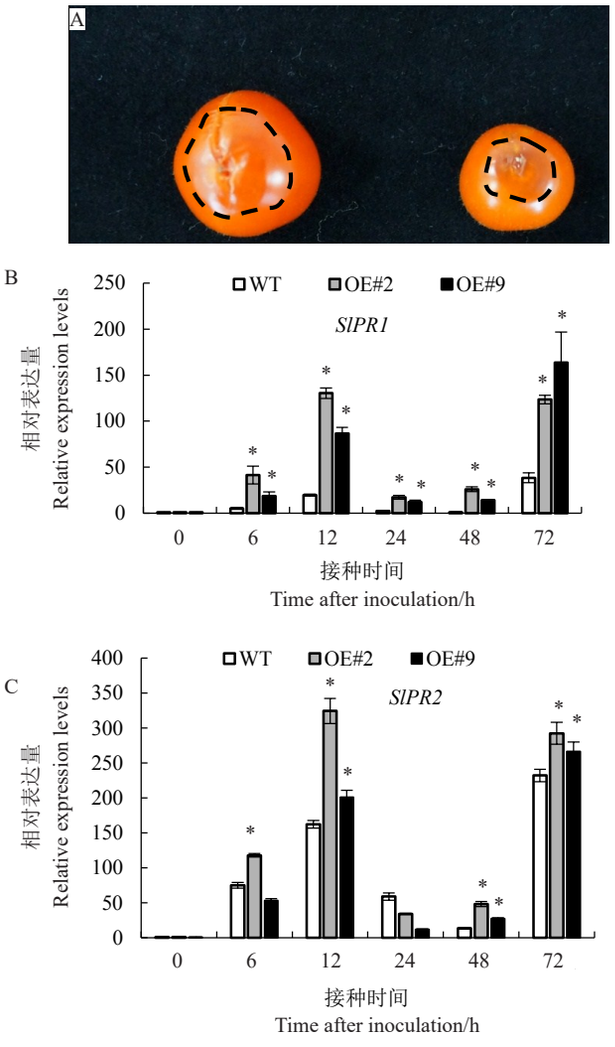
Fig. 5　Verification and phenotypes of wild type tomato and *VdMAPK7* overexpression tomato plants

实接种尖胞炭疽菌（*C. acutatum*），72 h 后，相对于野生型番茄果实中的病斑，*VdMAPK7* 转基因番茄果实病斑较小，发病症状较轻（图 6-A）。为更好阐释 *VdMAPK7* 转基因番茄果实接种炭疽菌后病斑较小现象，将野生型和 *VdMAPK7* 转基因番茄果实接种炭疽菌，接种后 0、6、12、24、48 和 72 h 采集番茄果皮，提取 RNA 反转录后，以 *SlActin* 为内参基因，分析 *VdMAPK7* 接种炭疽菌后不同时间段 *SlPR1* 和 *SlPR2* 的表达量变化。结果表明，相对于野生型番茄果实，*VdMAPK7* 转基因番茄果实中 *SlPR1* 基因的表达量在接种炭疽菌后各时间段均显著升高（图 6-B）；转基因番茄果实 *SlPR2* 基因表达量在接种 6、12、48 和 72 h 后显著升高（图 6-C）。这些结果说明过表达 *VdMAPK7* 基因的番茄果实对炭疽菌的抗性增强。

3　讨　论

MAPK 是一类丝氨酸/苏氨酸蛋白激酶，广泛存在于植物之中，位于 MAPK 级联途径中最下游，参与生物胁迫[24]。植物受到生物胁迫时，MAPK 信号通路被激活形成免疫应答，是植物产生抗病反应的早期信号[25]。

本研究中，葡萄炭疽病菌接种刺葡萄福安，*VdMAPK7* 基因在接种后第 7 天表达量最高，说明 *VdMAPK7* 基因可以响应葡萄炭疽菌而诱导表达。抗、感炭疽病的茶树品种转录组测序结果表明 MAPK 被 *R* 基因激活并介导了下游的抗病反应[26]。欧洲葡萄中共有 12 个 *MAPK* 基因，其中 *VvMPK1* 与 *VvMPK10* 基因受到活体寄生菌白粉菌诱导后表达[16]，葡萄中 *MAPK* 基因与死体营养型的葡萄炭疽病菌之间的关系未见报道。本研究中，*VdMAPK7* 转基因番茄植株表现为植株矮小，果实变小。这可能是由于基因过表达之后，影响转基因植株的正常代谢，从而影响转基因植株的生长发育，表现为矮化、弱小，这在别的研究中已有报道[27]。SA 作为植物激



A. 番茄果实接种炭疽菌 72 h 后表型(左为野生型,右为转基因型);B~C. 接种炭疽菌 0、6、12、24、48 和 72 h 后转基因株系和野生型番茄果实 *SlPR1*(B)和 *SlPR2*(C)的相对表达量。试验重复 3 次,每个值代表的是平均值±*SE*(*p<0.05)。

A. Symptom on fruits of wild type (left) and transgenic *VdMAPK7* (right) at 72 h after inoculation with *C. acutatum*; B-C. Expression levels of *SlPR1* (B) and *SlPR2* (C) at 0, 6, 12, 24, 48 and 72 h after inoculation. Each value represents the means±SE of three different experiments (*p<0.05).

图 6 过表达 *VdMAPK7* 转基因番茄果实对炭疽菌抗性的比较

Fig. 6 Assessment of resistance to *C. acutatum* in *VdMAPK7*-overexpressing tomato fruits

素,同时也是信号分子,在植物抗病反应过程中发挥重要功能,SA信号通路多参与活体营养型的病原菌应答,而JA途径则参与死体营养型的抗病反应[7]。本研究中,通过对转基因果实接种番茄炭疽病菌,病斑的面积相较于野生型番茄变小。这可能是由于过表达 *VdMAPK7* 基因后,番茄的抗病基因表达量升高,对炭疽病菌抗性增强。为了进一步验证转基因番茄果实中的抗病基因的表达量,研究中进行了qRT-PCR分析,转基因番茄果实中 *SlPR1* 和 *SlPR2* 基因表达量均显著高于野生型。有研究发现,*PR* 基因在植物抗病过程中,不仅参与了对活体寄生菌的抗性反应,还参与了对死体营养型病原菌的抗性反应。例如,Ma 等[28]证实,葡萄中 PR10.1 通过结合 VpVDAC 激活 ROS 通路,来抑制葡萄霜霉菌的侵染;Xie 等[23]对过表达 *VqDUF642* 的转基因番茄接种灰霉病后发现,*SlPR1*、*SlPR2*、*SlPR3* 和 *SlPR4* 基因均诱导表达。综合 *VdMAPK7* 基因可以响应葡萄炭疽菌的表达,以及过表达 *VdMAPK7* 的转基因番茄果实接种炭疽菌的表型和抗病基因的定量分析结果,说明 *VdMAPK7* 基因可以增强番茄对炭疽菌的抗性,并推测 *VdMAPK7* 基因参与葡萄对炭疽菌的抗病过程。

4 结 论

探究了刺葡萄 *VdMAPK7* 基因在葡萄抗炭疽菌中的作用。刺葡萄 *VdMAPK7* 基因可以响应炭疽菌诱导后表达量逐渐升高,在接种的第7天达到高峰;*VdMAPK7* 基因在番茄中过量表达均可增强对尖孢炭疽菌的抗性,推测 *VdMAPK7* 基因参与了葡萄对炭疽菌的胁迫响应,对定向改良葡萄抗病性提供了一定的参考依据。

参考文献 References:

[1] ICHIMURA K, SHINOZAKI K, TENA G, SHEEN J, HENRY Y, CHAMPION A, KREIS M, ZHANG S, HIRT H, WILSON C, HEBERLE-BORS E, ELLIS B E, MORRIS P, INNES R, ECKER J R, SCHEEL D K, KLESSIG D, MACHIDA Y, MUNDY J, OHASHI Y, WALKER J C. Mitogen-activated protein kinase cascades in plants: A new nomenclature[J]. Trends in Plant Science, 2002, 7(7): 301-308.

[2] SHI Y, HAN G H, WU H L, YE K, TIAN Z, WANG J, SHI H, YE M, ZOU H, HUO K. Casein kinase 2 interacts with human mitogen- and stress-activated protein kinase MSK_1 and phosphorylates it at multiple sites[J]. BMB Reports, 2009, 42(12): 840-845.

[3] JONAK C, KIEGERL S, LIGTERINK W, BARKER P J, HUSKISSON N S, HIRT H. Stress signaling in plants: A mitogen-activated protein kinase pathway is activated by cold and drought[J]. Proceedings of the National Academy of Sciences of the United States of America, 1996, 93(20): 11274-11279.

[4] BECKERS G J M, JASKIEWICZ M, LIU Y D, UNDERWOOD W R, HE S Y, ZHANG S, CONRATH U. Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana*[J]. The Plant Cell, 2009, 21(3):

944-953.

[5] ZIPFEL C，ROBATZEK S，NAVARRO L，OAKELEY E J，JONES J D G，FELIX G，BOLLER T. Bacterial disease resistance in *Arabidopsis* through flagellin perception[J]. Nature，2004，428(6984)：764-767.

[6] ANDREASSON E，ELLIS B. Convergence and specificity in the *Arabidopsis* MAPK nexus[J]. Trends in Plant Science，2010，15(2)：106-113.

[7] LÓPEZ M A，BANNENBERG G，CASTRESANA C. Controlling hormone signaling is a plant and pathogen challenge for growth and survival[J]. Current Opinion in Plant Biology，2008，11(4)：420-427.

[8] HAMEL L P，NICOLE M C，SRITUBTIM S，MORENCY M J，ELLIS M，EHLTING J，BEAUDOIN N，BARBAZUK B，KLESSIG D，LEE J，MARTIN G，MUNDY J，OHASHI Y，SCHEEL D，SHEEN J，XING T，ZHANG S，SEGUIN A，ELLIS B E. Ancient signals：Comparative genomics of plant *MAPK* and *MAPKK* gene families[J]. Trends in Plant Science，2006，11(4)：192-198.

[9] REYNA N S，YANG Y N. Molecular analysis of the rice MAP kinase gene family in relation to *Magnaporthe grisea* infection[J]. Molecular Plant-Microbe Interactions，2006，19(5)：530-540.

[10] NICOLE M C，HAMEL L P，MORENCY M J，BEAUDOIN N，ELLIS B E，SÉGUIN A. MAP-Ping genomic organization and organ-specific expression profiles of poplar MAP kinases and MAP kinase kinases[J/OL]. BMC Genomics，2006，7：223. DOI：10.1186/1471-2164-7-223.

[11] KONG X P，PAN J W，ZHANG D，JIANG S，CAI G，WANG L，LI D. Identification of mitogen-activated protein kinase kinase gene family and MKK-MAPK interaction network in maize[J]. Biochemical and Biophysical Research Communications，2013，441(4)：964-969.

[12] ZHANG S Z，XU R R，LUO X C，JIANG Z，SHU H. Genome-wide identification and expression analysis of *MAPK* and *MAPKK* gene family in *Malus domestica*[J]. Gene，2013，531(2)：377-387.

[13] ZHANG S，KLESSIG D F. Salicylic acid activates a 48-kD MAP kinase in tobacco[J]. The Plant Cell，1997，9(5)：809-824.

[14] ZHANG X T，CHENG T C，WANG G H，YAN Y，XIA Q. Cloning and evolutionary analysis of tobacco *MAPK* gene family[J]. Molecular Biology Reports，2013，40(2)：1407-1415.

[15] CHANG X L，NICK P. Defence signalling triggered by Flg22 and Harpin is integrated into a different stilbene output in *Vitis* cells[J/OL]. PLoS ONE，2012，7(7)：e40446. DOI：10.1371/journal.pone.0040446.

[16] 王刚 . 葡萄 MAPK 类基因家族的鉴定、表达分析及部分基因的功能验证[D]. 南京：南京农业大学，2014.
WANG Gang. Identification，expression analysis of mapk-like gene families in grapevine and functional characteration of some members[D]. Nanjing：Nanjing Agricultural University，2014.

[17] JIAO Y T，WANG D，WANG L，JIANG C，WANG Y. *VqMAPKKK38* is essential for stilbene accumulation in grapevine[J]. Horticulture Research，2017，4(1)：95-103.

[18] 朱自果，张庆田，李秀杰，韩真，李桂荣，李勃 . 燕山葡萄 MAPK 基因的克隆、亚细胞定位及表达分析[J]. 农业生物技术学报，2020，28(3)：429-440.
ZHU Ziguo，ZHANG Qingtian，LI Xiujie，HAN Zhen，LI Guirong，LI BO. Cloning，subcellular localization and expression analysis of MAPK genes from *Vitis yeshanesis*[J]. Journal of Agricultural Biotechnology，2020，28(3)：429-440.

[19] 贺普超，任治邦 . 我国葡萄属野生种对炭疽病抗性的研究[J]. 果树科学，1990，7(1)：7-12.
HE Puchao，REN Zhibang. Study on the resistances of wild *Vitis* species native to China to grape ripe rot[J]. Journal of Fruit Science，1990，7(1)：7-12.

[20] 雷龑，林雄杰，陈婷，蔡盛华，范国成 . 福建葡萄炭疽病病原鉴定及致病性分析[J]. 果树学报，2014，31(6)：1123-1127.
LEI Yan，LIN Xiongjie，CHEN Ting，CAI Shenghua，FAN Guocheng. Pathogen identification and pathogenicity analysis of grape ripe rot in Fujian[J]. Journal of Fruit Science，2014，31(6)：1123-1127.

[21] REID K E，OLSSON N，SCHLOSSER J，PENG F，LUND S T. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development[J/OL]. BMC Plant Biology，2006，6：27. DOI：10.1186/1471-2229-6-27.

[22] RAO X Y，HUANG X L，ZHOU Z C，LIN X. An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis[J]. Biostatistics，Bioinformatics and Biomathematics，2013，3(3)：71-85.

[23] XIE X Q，WANG Y J. VqDUF642，a gene isolated from the Chinese grape *Vitis quinquangularis*，is involved in berry development and pathogen resistance[J]. Planta，2016，244(5)：1075-1094.

[24] ZHANG S Q，KLESSIG D F. MAPK cascades in plant defense signaling[J]. Trends in Plant Science，2001，6(11)：520-527.

[25] MELKSHAM K J，WECKERT M A，STEEL C C. An unusnal bunch rot of grapes in sub-tropical regions of Anstralia caused by *Colletotrichum acutatum*[J]. Australasian Plant Pathology，2002，31(2)：193-194.

[26] WANG Y，HAO X，LU Q，WANG L，QIAN W，LI N，DING C，WANG X，YANG Y. Transcriptional analysis and histochemistry reveal that hypersensitive cell death and H_2O_2 have crucial roles in the resistance of tea plant (*Camellia sinensis* (L.) O. Kuntze) to anthracnose[J/OL]. Horticulture Research，2018，5：18. DOI：10.1038/s41438-018-0025-2.

[27] THALHAMMER A，BRYANT G，SULPICE R，HINCHA D K. Disordered cold Regulated15 proteins protect chloroplast membranes during freezing through binding and folding，but do not stabilize chloroplast enzymes *in vivo*[J]. Plant Physiology，2014，166(1)：190-201.

[28] MA H，XIANG G Q，LI Z Q，WANG Y，DOU M，SU L，YIN X，LIU R，WANG Y，XU Y. Grapevine VpPR10.1 functions in resistance to *Plasmopara viticola* through triggering a cell death-like defence response by interacting with VpVDAC3[J]. Plant Biotechnology Journal，2018，16(8)：1488-1501.