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# 葡萄VvWRKY5转录因子正调控*VvNPR1*提高白腐病抗病性

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**摘 要:【目的】**探究WRKY转录因子VvWRKY5在葡萄响应白腐病过程中的功能,并解析其调控抗病性的分子机制,为葡萄抗病分子育种提供理论依据。**【方法】**以红地球葡萄(*Vitis vinifera* 'Red Globe')为试材,克隆*VvWRKY5*(GSVIVT01019419001)并构建系统进化树;以*VvWRKY5*瞬时表达的葡萄叶片和稳定转化的葡萄愈伤组织为材料,通过白腐病菌接种试验,探究*VvWRKY5*是否调控葡萄对白腐病的抗性;通过qRT-PCR分析其对关键抗病基因表达水平的调控;并利用酵母单杂交和萤光素酶报告试验研究VvWRKY5与*VvNPR1*(nonexpressor of pathogenesis-related genes 1)启动子的结合及对其转录活性的影响。**【结果】***VvWRKY5*的开放阅读框为972 bp,编码323个氨基酸,预测的蛋白质分子质量是35.70 kD,等电点为5.85,含有1个WRKY保守结构域和C2H2锌指结构域。系统进化树分析表明,VvWRKY5与拟南芥AtWRKY22同源性最高。*VvWRKY5*在葡萄各组织中均有表达,其中,叶片和果实是最主要的表达部位。亚细胞定位显示VvWRKY5蛋白定位于细胞核中。功能分析表明,过表达*VvWRKY5*的葡萄叶片和愈伤组织的发病面积明显小于对照组,受伤害程度较轻,并能促进抗病关键基因*VvNPR1*(水杨酸免疫信号转导途径核心基因)和*VvPR1*(pathogenesis-related protein 1,病程防御相关蛋白1)的表达。相反,沉默*VvWRKY5*的葡萄叶片的发病面积明显大于对照组,病害程度较重,*VvNPR1*和*VvPR1*的表达量低于对照。酵母单杂交和萤光素酶报告试验发现,VvWRKY5能够与*VvNPR1*启动子结合并激发其转录活性。**【结论】**VvWRKY5可能通过提高*VvNPR1*的转录活性进而增强葡萄对白腐病的抗性。

**关键词:**葡萄;白腐病;WRKY转录因子;*VvNPR1*

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## VvWRKY5 transcription factor positively regulates *VvNPR1* to enhance white rot resistance in grape

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**Abstract:**【Objective】Grapevine (*Vitis vinifera* L.) is one of the most economically important fruit crops globally, valued for its use in fresh consumption, winemaking, and processed products. However, its productivity and quality are severely threatened by grape white rot, a devastating fungal disease caused by *Coniella diplodiella* (Speg.) Sacc (*C. diplodiella*). This pathogen primarily infects berries, leaves, and shoots, leading to fruit rot rates exceeding 50% in severe outbreaks and significant economic losses. Current management strategies rely heavily on chemical fungicides, which pose risks of environmental pollution, pathogen resistance, and food safety concerns. Thus, identifying host resistance genes and elucidating their molecular mechanisms is critical for developing sustainable disease management strategies through molecular breeding. Plant transcription factors (TFs) play pivotal roles in regulating immune responses by orchestrating the expression of defense-related genes. The WRKY family,

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characterized by the conserved WRKYGQ motif and zinc finger domains, is well-documented for its involvement in biotic stress responses, but their functions in grape white rot remain poorly understood. Here, we cloned and characterized *VvWRKY5* (GSVIVT01019419001), a WRKY TF gene from the grape cultivar Red Globe, to investigate its role in white rot resistance and underlying regulatory mechanisms.【Methods】A phylogenetic tree was constructed using MEGA7.0 with the neighbor-joining method (1000 bootstrap replicates), including VvWRKY5 and WRKY proteins from *Arabidopsis*, *Oryza sativa*, and *Malus domestica*. For subcellular localization, the coding sequence (CDS) of *VvWRKY5* was fused to GFP in the pRI101 vector and transiently expressed in onion epidermal cells via *Agrobacterium tumefaciens*-mediated transformation. Fluorescence was observed using a confocal laser scanning microscope. To identify the potential roles of VvWRKY5 in resistance to white rot, *Agrobacterium tumefaciens* carrying recombinant plasmids (VvWRKY5-pRI and VvWRKY5-TRV2) were infiltrated into Red Globe leaves and Red Gamay grape callus, respectively, and their resistance was subsequently evaluated following inoculating the *C. diplodiella*. Subsequently, we observed the phenotypes of *VvWRKY5*-transgenic and non-transgenic materials after inoculation with the white rot pathogen, measured stress-related physiological parameters (including $O_2^-$ and MDA contents, etc.), and analyzed the expression levels of the key stress response genes *VvNPR1* (nonexpressor of pathogenesis-related genes 1, a core gene in the salicylic acid signal transduction pathway) and *VvPR1* (encoding pathogenesis-related protein 1) using qRT-PCR technology. A yeast one-hybrid was performed to validate the interaction between VvWRKY5 and *VvNPR1* promoter by observing the growth status of the yeast cells cotransformed with VvWRKY5-pGADT7 and *ProVvNPR1*-pHIS2 on the SD/-Trp/-His/-Leu defined medium supplemented with the 3-amino-1, 2, 4-triazole (3-AT). In addition, the effect of VvWRKY5 on *VvNPR1* transcriptional activity was studied using the luciferase reporter assay. The coding region sequence of *VvWRKY5* was inserted into the pRI101-AN vector and the promoter fragment of *VvNPR1* was introduced into pGreenII 0800-LUC vector. One-month-old tobacco (*N. benthamiana*) leaves were infected with *Agrobacterium tumefaciens* strain GV3101 carrying the recombinant plasmids. After the plants were kept in the dark for 48 h, luciferase activity was detected using the live imaging analysis system.【Results】Sequence analysis indicated that the open reading frame of *VvWRKY5* was 972 bp, which encoded 323 amino acids. The predicted molecular weight of the protein was 35.70 kD, the theoretical pI was 5.85, and the protein contained a WRKY conserved domain and a C2H2 zinc finger domain. Phylogenetic analysis showed VvWRKY5 shared 78% amino acid identity with AtWRKY22 from *Arabidopsis thaliana*, indicating a close evolutionary relationship. qRT-PCR results showed that *VvWRKY5* expression was highly induced after *C. diplodiella* inoculation and was ubiquitously expressed in all tissues, with the highest expression levels in leaves (3.0-fold higher than roots) and fruits (2.7-fold higher than roots). Subcellular localization assays confirmed that VvWRKY5-GFP fluorescence was exclusively localized to the nucleus, consistent with its role as a TF. Functional analysis indicated that the lesion area of grape leaves and callus overexpressing *VvWRKY5* was significantly smaller than that of the control, the disease severity was reduced, and it could promote the expression of key disease resistance genes *VvNPR1* and *VvPR1*. On the contrary, the lesion area of grape leaves with *VvWRKY5* silenced was significantly larger than the control, the disease severity was significantly higher and the expression levels of *VvNPR1* and *VvPR1* were lower than the control. Yeast one-hybrid assay demonstrated that VvWRKY5 directly bound to the *VvNPR1* promoter containing a W-box motif (-270 bp), as yeast cells co-transformed with bait (*ProVvNPR1*-pHIS2) and prey (VvWRKY5-pGADT7) plasmids grew well on selective medium. Furthermore, the luciferase reporter assay revealed that VvWRKY5 could promote

the transcriptional activity of *VvNPR1*, as the fluorescence signal in the tobacco leaves co-injected with 35S::VvWRKY5 and *ProVvNPR1*-LUC (35S::VvWRKY5 + ProVvNPR1-LUC) was significantly stronger than the control groups (35S + LUC, 35S::VvWRKY5 + LUC, 35S + *ProVvNPR1*-LUC).【Conclusion】In conclusion, VvWRKY5 is a typical WRKY transcription factor associated with resistance to white rot in grape, which is significantly upregulated under *C. diplodiella* induction, and its overexpression positively regulates the resistance of grape to *C. diplodiella*. Moreover, its nuclear localization and tissue-specific expression (highest in leaves/fruits) align with its role in defending against foliar and fruit pathogens. Additionally, this study reveals a novel mechanism by which VvWRKY5 enhances grape resistance to white rot through activation of VvNPR1 transcription. This expands our understanding of WRKY-mediated immunity in grape and provides a candidate gene for breeding disease-resistant cultivars.

**Key words:** Grape; White rot; WRKY transcription factor; *VvNPR1*

葡萄白腐病是由白腐垫壳孢[*Coniella diplodiella*(Speg.)Sacc.]引起的真菌性病害，是世界范围内葡萄种植区的毁灭性病害之一，严重威胁产业的发展[1-2]。植株感染病害后，叶片边缘出现不规则的水渍状病斑并逐渐扩大成同心轮纹状的褐色病斑；葡萄果粒逐渐变褐变软直至果穗腐烂，造成大面积减产[3]。近年来，受葡萄种植密度和雨水等因素的综合影响，葡萄白腐病呈现出发病提前并逐年加重的趋势[4-5]。生产中，防治葡萄白腐病仍以化学药剂为主，但该策略不仅显著增加果园管理成本，其导致的果实农药残留与生态环境污染还会直接威胁人体健康。转录因子作为植物胁迫响应网络中的核心调控因子，在介导植物对生物和非生物胁迫的抗性反应中发挥关键作用[6-7]。因此，挖掘葡萄白腐病抗病相关基因资源、解析其分子调控机制、定向改良葡萄抗病性，是当前葡萄产业中亟待解决的核心科学问题与绿色生产需求。

WRKY转录因子家族是植物特有的超级转录因子家族之一。大量研究表明，WRKY转录因子可通过与靶基因启动子区域的W-box元件(TTGACC/T)特异性结合，精准调控下游防御相关基因的表达，进而参与植物对病原菌的免疫应答[8]。在一些模式植物与作物中，WRKY转录因子的抗病功能已得到广泛验证。拟南芥中，过表达*AtWRKY33*提高了植株对灰霉菌的抗性[9]；异源过表达草莓*FvWRKY42*的拟南芥增强了对白粉病的抗性[10]。柑橘中，*CsWRKY22*基因的表达水平受柑橘溃疡病菌侵染诱导显著上调，且在锦橙中超量表达*CsWRKY22*显著增强了植株对柑橘溃疡病的感病性[11]。苹果中，*MdWRKY17*过表达能够降低苹果炭疽病的发病程度[12]；*MdWRKY100*过表达能提高转基因苹果对炭疽病的耐受性[13]；MdWRKY1通过调控病程相关蛋白PR1介导的免疫平衡来增强苹果对白粉病的抗性[14]。水稻中，OsWRKY03定位于抗病基因*OsNPR1*的上游，能够激活*OsNPR1*的表达，从而参与水杨酸依赖的防御反应[15]；OsWRKY68通过*Xa21*正调控水稻对白叶枯病的抗性反应[16]。然而，葡萄WRKY转录因子的抗病功能研究目前主要聚焦于白粉病：毛葡萄VqWRKY46通过诱导过敏性细胞死亡、促进胼胝质积累和上调防御相关基因(*NPR1*、*PR5*、*CAT1*)表达，显著增强植株对白粉病的抗性[17]；此外，VqWRKY6和VqWRKY31也均被鉴定为葡萄白粉病抗性的正调节因子，其中VqWRKY6通过促进活性氧(ROS)爆发激活氧化应激防御[18-19]。尽管WRKY转录因子在葡萄白粉病研究中已取得了一定进展，但其在葡萄抗白腐病中的功能解析却鲜有报道。

笔者前期通过对白腐菌接种后0、24、48、72 h的葡萄叶片进行转录组数据[20]分析发现，*VvWRKY5*基因的表达受病原菌诱导显著上调。为进一步探究该基因的功能及其抗病机制，本研究以红地球葡萄为试材，通过对农杆菌介导的瞬时转化的葡萄叶片和稳定过表达的葡萄愈伤组织中进行接菌试验，结合胁迫生理指标和防御相关基因表达量的测定，发现VvWRKY5正调控葡萄对白腐病原菌的抗性。同时，利用酵母单杂交和萤光素酶报告等试验对VvWRKY5的抗病机制进行了进一步研究，以期揭示VvWRKY5调控葡萄白腐病抗性的分子机制，为

葡萄的抗性育种研究提供基因资源和理论依据。

# 1　材料和方法

## 1.1　试验材料

供试材料采自沈阳农业大学葡萄实验园（北纬41°50′，东经123°24′）的红地球葡萄（*Vitis vinifera* ‘Red Globe’）的叶片。采样选取大小一致、健康无病害的叶片60枚，分成3份，每20枚叶片作为1次重复，用于瞬时转化试验、*VvWRKY5*基因的克隆和表达分析。

用于稳定遗传转化的愈伤组织为佳美葡萄（*Vitis vinifera* ‘Red Gamay’），于无菌培养皿中进行愈伤组织的培养，每隔20 d进行1次继代。

本试验所用的葡萄白腐病病原菌（编号：WR01）由中国农业科学院郑州果树研究所张颖老师馈赠。

本氏烟草（*Nicotiana benthamiana*）在25 ℃的光照培养箱中进行培养，光周期为光照16 h /黑暗8 h。

## 1.2　RNA提取与基因表达分析

RNA提取和实时荧光定量PCR（qRT-PCR）参照Yin等[18]的方法。叶片总RNA提取采用康为世纪的RNA prep pure Plant Kit试剂盒完成。cDNA的合成采用PrimeScript™ RT reagent Kit试剂盒（宝生物，大连）完成。使用在线软件Primer 5.0设计qRT-PCR的引物（表1）。以*VvActin*为内参基因，采用ABI 7500荧光定量PCR仪（Applied Biosystems，USA）进行试验。反应体系为：UltraSYBR Green Mixture reagent 10.0 μL，dd$H_2O$ 7.0 μL，上、下游引物（10 μmol·$L^{-1}$）各1.0 μL，cDNA 1.0 μL。反应程序为：95 ℃预变性10 min；95 ℃变性10 s，60 ℃退火30 s，72 ℃延伸32 s，40个循环。基因的相对表达量采用$2^{-\Delta\Delta CT}$算法，每个样本设置3次重复。

## 1.3　基因克隆分析

利用DNAMAN软件设计*VvWRKY5*（基因ID：GSVIVT01019419001）基因扩增的引物（表1），参考基因序列来源于12X *V. vinifera* cv. Pinot noir genome（quasi-homozygous line PN40024，网址http://www.phytozome.net）。以红地球葡萄叶片的cDNA和DNA为模板分别对基因和启动子进行扩增。PCR反应条件为：98 ℃预变性30 s；98 ℃变性10 s，

**表1　基因克隆和qRT-PCR所用引物序列**

**Table 1　Primers used for cloning and qRT-PCR analysis**

| 引物名称<br>Primer name | 序列（5′–3′）<br>Sequence (5′–3′) | 用途<br>Purpose |
|---|---|---|
| *VvWRKY5*-F | TGTGGGCTTGGCGTAAATA | 实时荧光定量PCR<br>qRT-PCR |
| *VvWRKY5*-R | GGCGAGTTGGATGAGAATGG | |
| *VvNPR1*-F | CTACAGAGGAGCGACTTC | |
| *VvNPR1*-R | TGGATGATGATGAGGATGAT | |
| *VvPR1*-F | CATTGACAGGCACTGATG | |
| *VvPR1*-R | CCTCCATTGTTGCATTGAA | |
| *VvActin*-F | GATTCTGGTGATGGTGTGAGT | |
| *VvActin*-R | GACAATTTCCCGTTCAGCAGT | |
| *VvWRKY5*-GFP-F | GTCGACATGGGGGACTGGGATTTGC | 亚细胞定位<br>Subcellular localization |
| *VvWRKY5*-GFP-R | GGATCCGCAACCGCCGGCGGC | |
| *VvWRKY5*-pTRV2-F | GGATCCGTGACCTACACAGCTGAACAC | 瞬时转化葡萄叶片<br>Transient transformation of grape leaves |
| *VvWRKY5*-pTRV2-R | CCCGGGTCAGCAACCGCCGGCGGC | |
| *VvWRKY5*-pRI-F | GTCGACATGGGGGACTGGGATTTGC | |
| *VvWRKY5*-pRI-R | GGATCCGCAACCGCCGGCGGC | |
| *35S::VvWRKY5*-F | GTCGACATGGGGGACTGGGATTTGC | 萤光素酶报告试验<br>Luciferase reporter assay |
| *35S::VvWRKY5*-R | GGATCCGCAACCGCCGGCGGCAGTTG | |
| *ProVvNPR1*-LUC-F | AAGCTTATGTTCCAAATTGTTTGCTTG | |
| *ProVvNPR1*-LUC-R | GGATCCGGAGCCGAGAGACAAAAAGG | |
| *VvWRKY5*-AD-F | CCCGGGATGGACTACA GAGCTGCTCT | 酵母单杂交<br>Yeast one-hybrid assay |
| *VvWRKY5*-AD-R | GTCGACTTACTTCTTGCAAGAGAGTCTAC | |
| *ProVvNPR1*-pHIS2-F | GAATTCCTCGCAACTCTTATCATCTAGG | |
| *ProVvNPR1*-pHIS2-R | GAGCTCGGAGCCGAGAGACAAAAAG | |

58 ℃退火30 s，72 ℃延伸45 s，35个循环；72 ℃延伸10 min。PCR产物用1%琼脂糖凝胶分离并回收目的条带。连接T1零背景载体（全式金，北京），转化大肠杆菌DH5α，选取阳性克隆由生工生物工程（上海）股份有限公司进行测序分析。

### 1.4 进化树分析与结构域预测

从NCBI（https://www.ncbi.nlm.nih.gov/）数据库中下载代表性物种拟南芥（*Arabidopsis thaliana*）、水稻（*Oryza sativa*）和苹果（*Malus domestica*）中的多个WRKY家族成员。利用MEGA 7.0软件，采用邻接法（Neighbor- Joining）构建VvWRKY5与其他WRKY蛋白的系统发育进化树，建树参数No. of Bootstrap Replications设置为1000。同时，利用SMART在线网站（http://smart.embl-heidelberg.de/）对VvWRKY5蛋白的功能结构域进行分析预测。

### 1.5 亚细胞定位观察

采用农杆菌瞬时侵染洋葱内表皮组织的方法进行VvWRKY5蛋白的亚细胞定位观察。具体操作步骤为：选取新鲜的紫皮洋葱，于无菌条件下剥离中部内表皮（约1 cm×1 cm），随后将其平铺于无抗生素的MS固体培养基上预培养2 d；将带有VvWRKY5-pRI101-GFP重组质粒的GV3101农杆菌菌株活化至$OD_{600}$=0.6~0.8，离心收集菌体并加入乙酰丁香酮（acetosyringone，AS，50 mg·mL$^{-1}$），配制成侵染液。28 ℃黑暗静置25 min后用于侵染洋葱内表皮；真空侵染10 min后，吸干表面多余液体，然后将洋葱内表皮平铺于MS固体培养基上，24 ℃黑暗共培养2~3 d；于培养结束后使用激光共聚焦显微镜（TCS SP8，徕卡，德国）观察荧光信号，同时以空载体pRI101-GFP侵染的洋葱内表皮作为阴性对照。

### 1.6 酵母单杂交

酵母单杂交参照张震等[21]的方法进行。利用同源重组法将*VvNPR1*基因ATG上游的启动子序列（2000 bp）连接到pHIS2载体上（Clontech），构成*ProVvNPR1*- pHIS2融合载体；与此同时，将*VvWRKY5*基因编码区序列连接至pGADT7载体，构成VvWRKY5-pGADT7融合载体。将连接好的重组质粒利用酵母转化试剂盒（宝生物，大连）转化到Y187酵母感受态细胞（唯地，上海）中，然后涂布于含有3-氨基-1,2,4-三唑（3-amino-1,2,4-triazole，3-AT）的SD/-Trp/-His/-Leu（SD/-T/-H/-L）缺陷型培养基上，在28 ℃培养箱中倒置培养2~3 d后观察酵母的生长情况，判断互作关系。酵母单杂交试验所用引物见表1。

### 1.7 萤光素酶报告试验

利用同源重组法将*VvWRKY5*的编码区序列连接到pRI101-AN载体上，并将*VvNPR1*启动子序列（2000 bp）连接至pGreenII0800-LUC载体。将携带35S::VvWRKY5和*ProVvNPR1*-LUC重组质粒的农杆菌菌株GV3101于YEP液体培养基中培养至$OD_{600}$为0.6~0.8，5000 r·min$^{-1}$离心收集菌体，并利用悬浮液（1 mol·L$^{-1}$ MES-KOH，1.0 mL；1 mol·L$^{-1}$ $MgCl_2$，1.0 mL；100 mmol·L$^{-1}$ AS，100.0 μL；dd$H_2O$，98.0 mL）悬浮菌体至$OD_{600}$为0.8~1.0，将菌液按需求等体积混匀后，于28 ℃培养箱中黑暗静置1.5 h。用去掉针头的1.0 mL无菌注射器将菌液缓慢注射于烟草的叶片背面，直至叶片出现深绿色水渍状。接种农杆菌的烟草于黑暗中培养48 h后，用植物活体成像系统（Tanon-5200，上海）观察荧光强度。荧光素酶活性测定使用碧云天荧光素酶活性检测试剂盒（Cat No. RG027）完成。

### 1.8 农杆菌介导的葡萄叶片瞬时转化及病原菌接种

将*VvWRKY5*的编码区序列连接到pRI101-AN载体以构建VvWRKY5-pRI重组质粒。与此同时，利用同源重组法将*VvWRKY5*序列连接到TRV2载体上，构建VvWRKY5-TRV2融合质粒，TRV1作为辅助质粒。利用冻融法将以上质粒导入农杆菌菌株EHA105，并于YEP液体培养基中培养至$OD_{600}$为0.6~0.8，5000 r·min$^{-1}$离心收集菌体，然后利用悬浮液悬浮菌体至$OD_{600}$为0.3~0.5，于28 ℃培养箱中黑暗静置3 h。将红地球叶片避开主叶脉，分割成直径为1.5 cm的叶盘，叶片正面朝上，浸没在装有农杆菌悬浮液的培养皿中进行真空渗滤20 min。用无菌水将叶片表面的菌液漂洗干净后，将其置于无菌水保湿的干净滤纸上，叶背面朝上。利用菌饼法（菌饼直径为0.2 cm）对瞬时转化的葡萄叶片接种白腐病病原菌。将接种后的叶片置于28 ℃、相对湿度为90%的光照培养箱中进行培养，2 d后拍照观察，收集叶片样品用于后续研究。

### 1.9 农杆菌介导的葡萄愈伤组织侵染及病原菌接种

将VvWRKY5-pRI重组质粒通过冻融法导入农杆菌菌株EHA105中，于YEP（含Rif和Kan抗生素）液体培养基中培养至$OD_{600}$为0.4~0.6，5000 r·min$^{-1}$

离心收集菌体，然后利用30 mL B5液体培养基（含有100 μmol·L$^{-1}$ AS）重悬菌体获得侵染液。将生长15 d左右的葡萄愈伤组织加入配制好的侵染液中，24 ℃、120 r·min$^{-1}$，振荡转化30 min。过滤愈伤组织并用无菌滤纸吸干表面水分后，将其平铺于无抗生素的B5琼脂固体培养基上2~3 d，然后将其转移至含巴龙霉素和卡那霉素的B5凝胶筛选培养基上30 d左右，直至抗性愈伤长出。愈伤组织的接菌试验同样采用菌饼法进行。

### 1.10　生理指标的测定

超氧阴离子（$O_2^-$）含量的测定采用苏州科铭公司的试剂盒完成。丙二醛（MDA）含量的测定参照Jiang等[22]的方法进行。具体来说，称取接菌处理后的葡萄叶片或愈伤组织0.1 g，加入4 mL提取液[20%三氯乙酸（*m*/*V*）和1%硫代巴比妥酸]，95 ℃水浴30 min，高速离心，取上清液用紫外分光光度计测定532 nm处的吸光值（上海普析）。每个试验进行3次独立的生物学重复。

### 1.11　数据分析

采用Prism软件进行数据分析并作图，使用SPSS 19.0软件对试验数据进行统计，并利用Student's *t*-test进行差异显著性分析（*$P$＜0.05，**$P$＜0.01）。

## 2　结果与分析

### 2.1　*VvWRKY5*基因的克隆

以红地球葡萄幼嫩叶片cDNA为模板，通过PCR扩增得到一条大小接近1000 bp的特异性条带（图1）。通过对该克隆片段进行胶回收和测序，发现该基因的开放阅读框为972 bp，编码323个氨基酸（Amino acid，aa），与参考序列（基因ID：GSVIVT01019419001）相似度100%，因此命名为*VvWRKY5*。利用Expasy在线分析系统对VvWRKY5蛋白的理化性质进行分析，结果显示该蛋白分子式为$C_{1571}H_{2430}N_{434}O_{495}S_{12}$，分子质量为35.70 kD，理论等电点为5.85。

### 2.2　*VvWRKY5*进化树、蛋白结构域和亚细胞定位分析

为探究VvWRKY5蛋白与不同物种同源蛋白的系统发育关系，本研究以代表性物种拟南芥、水稻和苹果中的多个WRKY家族成员为对象，采用邻接法构建了系统发育树。结果显示，VvWRKY5与拟南芥AtWRKY22蛋白聚为同一进化支，表明两者具有较高的序列相似性（图2-A）。利用SMART蛋白功能域预测数据库分析VvWRKY5蛋白的结构域，表明141~147 aa处含有一个保守的WRKY结构域，基序为WRKYGQK，以及在161~193 aa处含有一个C2H2类型的锌指结构域（图2-B）。为了研究VvWRKY5的亚细胞定位情况，通过农杆菌介导的瞬时转化法将VvWRKY5-GFP融合蛋白导入新鲜洋葱的内表皮细胞中。黑暗培养2 d后使用TCS SP8激光共聚焦显微镜进行亚细胞定位观察。结果如图2-C所示，对照35S::GFP蛋白弥散分布在整个细胞中，而VvWRKY5-GFP荧光则主要在细胞核中表达，表明VvWRKY5是一个核定位转录因子。

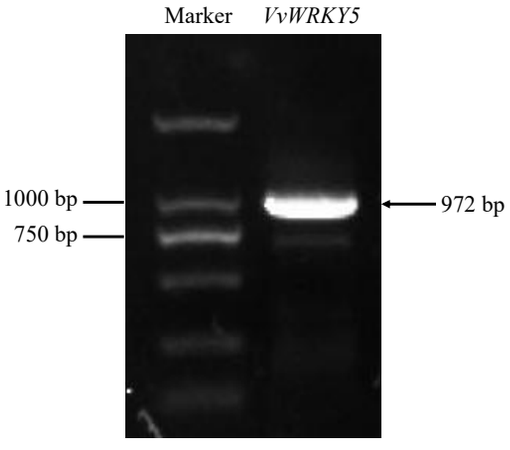



**图1　*VvWRKY5*基因RT-PCR产物**

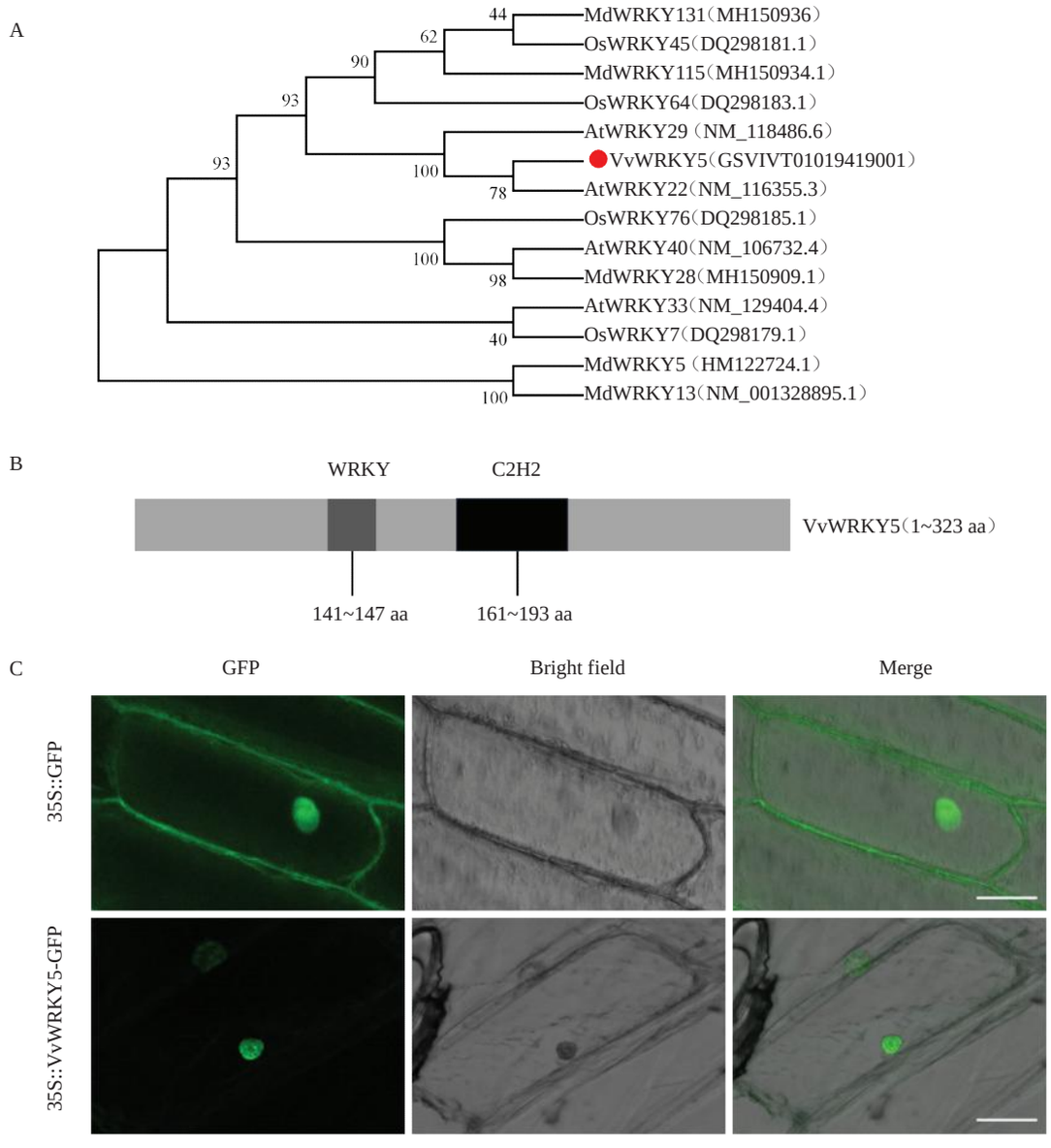
**Fig. 1　RT-PCR product of *VvWRKY5***

### 2.3　*VvWRKY5*的表达特性分析

为进一步了解*VvWRKY5*在葡萄抗病性中的潜在作用，利用qRT-PCR技术分析了在接种白腐病原菌后红地球葡萄离体叶片中*VvWRKY5*的转录水平。结果显示，白腐病原菌侵染后，*VvWRKY5*的转录水平显著升高，并于侵染第12 h时达到峰值，约为处理前的8倍（图3-A）。此外，笔者还分析了*VvWRKY5*在不同组织器官（根、茎、叶、卷须、果实）中的表达水平。如图3-B所示，*VvWRKY5*主要在叶片和果实中表达。

### 2.4　*VvWRKY5*转基因葡萄叶片接菌后的表型及胁迫指标的测定

通过农杆菌介导的侵染法，获得了*VvWRKY5*瞬时过表达（VvWRKY5-pRI）和沉默（VvWRKY5-TRV）的红地球葡萄叶片组织（图4-A，B），并利用菌饼法对其进行了白腐病菌接种试验。结果显示，接




A. VvWRKY5 系统发育进化树；B. VvWRKY5 保守结构域分析；C. VvWRKY5 亚细胞定位分析。At. 拟南芥；Os. 水稻；Md. 苹果。

A. Phylogenetic tree analysis of VvWRKY5; B. Conserved domain analysis of VvWRKY5; C. Subcellular localization analysis of VvWRKY5. At. *Arabidopsis thaliana*; Os. *Oryza sativa*; Md. *Malus domestica*.

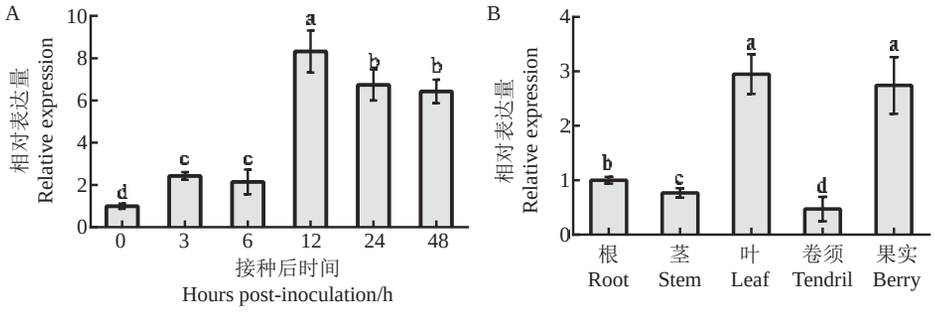
**图 2 VvWRKY5 蛋白系统进化树、保守结构域和亚细胞定位分析**

**Fig. 2 Phylogenetic tree analysis, structure prediction, and subcellular localization of VvWRKY5 protein**

种 2 d 后，VvWRKY5-pRI 葡萄叶片的发病面积要明显小于对照（pRI），叶片失绿较轻；相反，VvWRKY5-TRV 葡萄叶片的发病面积要明显大于对照（TRV），且叶片失绿严重（图 4-A，C）。进一步对胁迫相关生理指标进行测定，结果显示，VvWRKY5-pRI 葡萄叶片中的 $O_2^-$ 含量（图 4-D）、MDA 含量（图 4-E）和电解质渗透率（图 4-F）均显著低于 pRI；而 VvWRKY5-TRV 葡萄叶片中的 $O_2^-$ 含量、MDA 含量和电解质渗透率则均显著高于 TRV。以上结果表明，过表达 *VvWRKY5* 能够提高葡萄对白腐病的抗性。

### 2.5 *VvWRKY5* 稳定过表达葡萄愈伤组织接菌后的表型及胁迫指标的测定

为了进一步验证 *VvWRKY5* 在葡萄白腐病抗性中的作用，笔者还通过冻融法将 VvWRKY5-pRI101 融合质粒转化农杆菌 EHA105，以佳美葡萄愈伤组织为材料，进行稳定的遗传转化试验，获得了 VvWRKY5-OE#8 和 VvWRKY5-OE#11 两个独立的过表达株系（图 5-A，B）。如图所示，病原菌接种 2 d

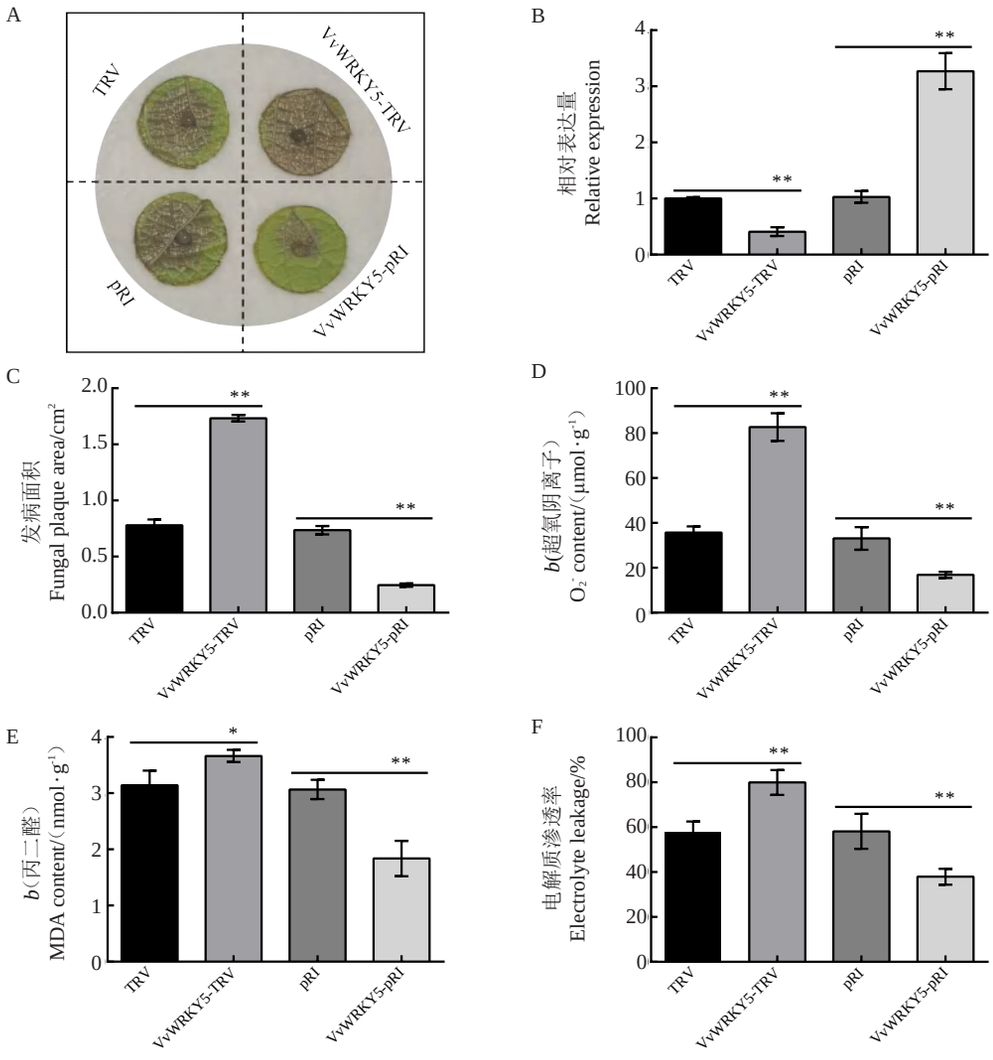



不同小写字母表示差异显著（ANOVA；$P<0.05$）。

Different small letters indicated significant differences (ANOVA; $P<0.05$).

**图3　*VvWRKY5*在白腐病病原菌侵染后（A）和葡萄不同组织中（B）的表达模式**

**Fig. 3　Expression pattern of *VvWRKY5* in response to *Coniella diplodiella* infection (A) and in various grapevine tissues (B)**




A. 叶片接种白腐菌后的表型；B. *VvWRKY5*相对表达量；C. 发病面积；D. 超氧阴离子含量；E. 丙二醛含量；F. 电解质渗透率。* 表示在 $P<0.05$ 水平下差异显著；** 表示在 $P<0.01$ 水平下差异极显著。

A. The phenotype of grape leaves after white rot inoculation; B. Relative expression of *VvWRKY5*; C. Fungal plaque area; D. $O_2^-$ content; E. MDA content; F. Electrolyte leakage. * indicates significant difference at $P < 0.05$; ** indicates extremely significant difference at $P < 0.01$.

**图4　瞬时过表达和沉默*VvWRKY5*的红地球葡萄叶片接种白腐菌后的表型及相关生理指标的测定**

**Fig. 4　The phenotype and related physiological indexes of Red Globe leaves with transient silence and overexpression of *VvWRKY5* after inoculation with white rot**

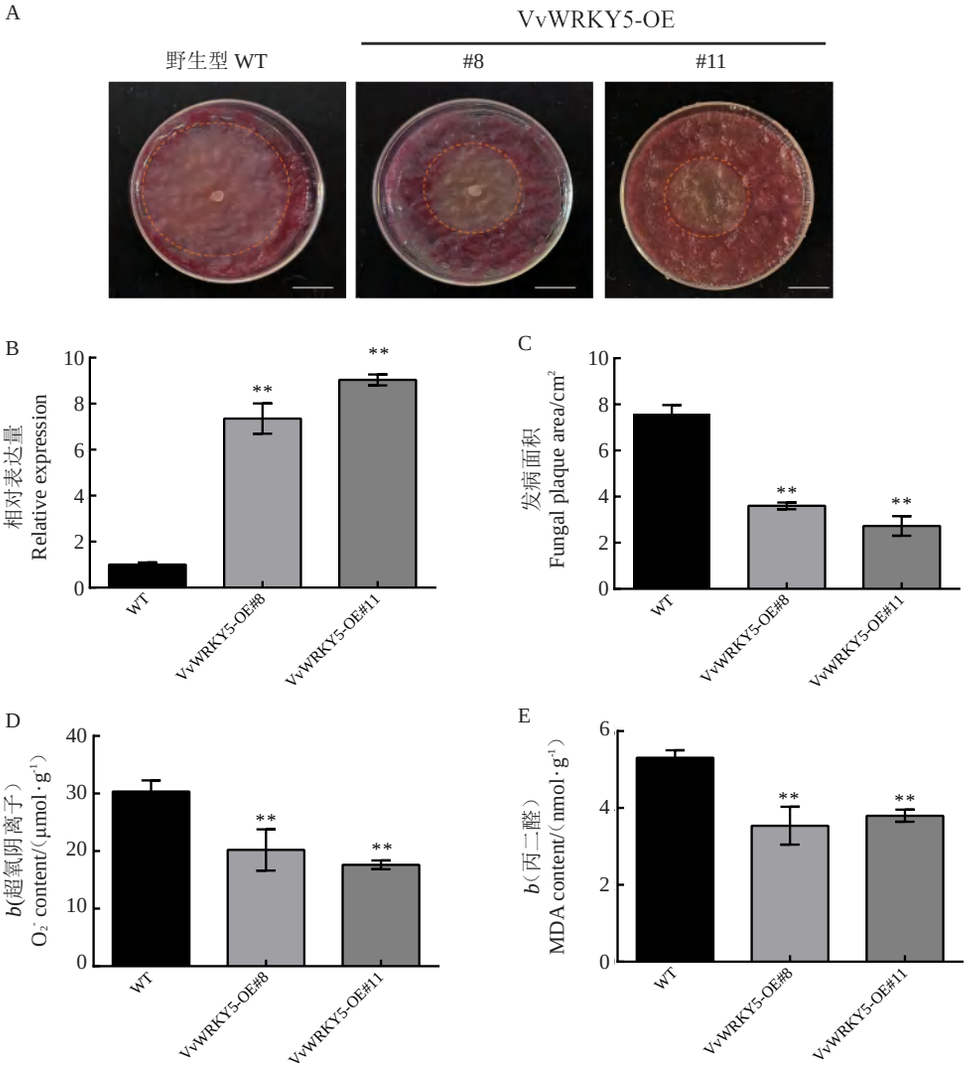
后，*VvWRKY5*过表达葡萄愈伤组织表现出比野生型（WT）更强的抗白腐病能力，具体表现为：菌丝扩散速度受到抑制，病斑面积明显小于WT（图5-A，C）；*VvWRKY5*过表达愈伤组织中$O_2^-$含量（图5-D）和MDA含量（图5-E）显著低于WT。以上结果表明，过表达*VvWRKY5*增强了葡萄愈伤组织对白腐病病原菌的抗性，与葡萄叶片中的研究结果一致。

### 2.6 *VvWRKY5*转基因葡萄叶片中防御相关基因的表达水平分析

利用qRT-PCR技术，对接种白腐病病原菌的*VvWRKY5*转基因葡萄叶片中的防御相关基因的表达量进行了检测。*VvNPR1*是水杨酸免疫信号转导途径中的核心基因，*VvPR1*编码病程防御相关蛋白1，两者均是植物抗病通路中的关键基因[15,17,23]。结果如图6所示，过表达*VvWRKY5*的葡萄叶片中*VvNPR1*和*VvPR1*的表达量均明显升高，而沉默*VvWRKY5*的葡萄叶片中*VvNPR1*和*VvPR1*的表达量均明显下降。其中，与*VvPR1*相比，*VvNPR1*基因受VvWRKY5的调控作用更为显著。

### 2.7 *VvWRKY5*结合*VvNPR1*启动子并提高其转录活性

为进一步探究VvWRKY5抗葡萄白腐病的分子




A. 葡萄愈伤组织接种白腐菌后的表型；B. *VvWRKY5* 相对表达量；C. 发病面积；D. 超氧阴离子含量；E. 丙二醛含量。比例尺为 1 cm。

A. The phenotype of grape callus after white rot inoculation; B. Relative expression of *VvWRKY5*; C. Fungal plaque area; D. $O_2^-$ content; E. MDA content. Scale bar=1 cm.

**图 5 过表达 *VvWRKY5* 葡萄愈伤组织接种白腐菌后的表型及相关生理指标的测定**

**Fig. 5 The phenotype and related physiological indexes of *VvWRKY5*-overexpressed grape callus after inoculation with white rot**

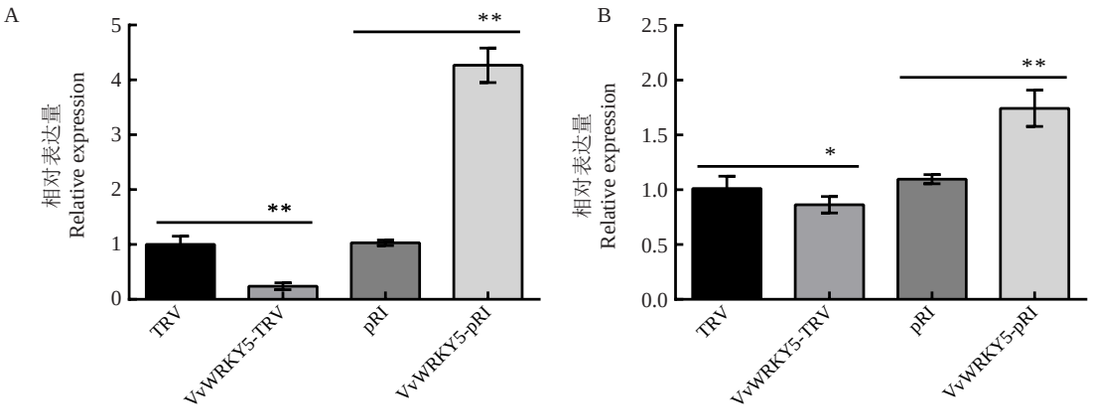



**图6　瞬时过表达和沉默*VvWRKY5*的葡萄叶片接种白腐菌后防御相关基因*VvNPR1*（A）和*VvPR1*（B）的表达水平分析**

**Fig. 6　Expression levels of resistance-related genes *VvNPR1* (A) and *VvPR1* (B) in grape leaves with transient silence and overexpression of *VvWRKY5* after inoculation with white rot**

机制，利用PlantCARE软件（http://bioinformatics.psb.ugent.be/webtools/plantcare/html/）对*VvNPR1*的启动子上的顺式作用元件进行了分析，发现*VvNPR1*启动子上含有响应光照、干旱胁迫等相关的元件，并且在ATG上游270 bp处含有一个WRKY转录因子结合的W-box位点（图7-A）。因此，笔者猜测VvWRKY5可能通过该位点与*VvNPR1*的启动子结合。

为了验证这一假设，笔者进行了酵母单杂交试验。结果如图7-B所示，共转化空载体pGADT7和*ProVvNPR1*-pHIS2的酵母感受态细胞在含有100 mmol·L$^{-1}$ 3-AT的SD/-T/-H/-L缺陷型培养基上无法生长；相反，共转化VvWRKY5-pGADT7和*ProVvNPR1*-pHIS2的酵母感受态细胞却能在含有100 mmol·L$^{-1}$ 3-AT的SD/-T/-H/-L培养基上正常生长，这表明VvWRKY5能够与*VvNPR1*的启动子在酵母细胞体内结合。

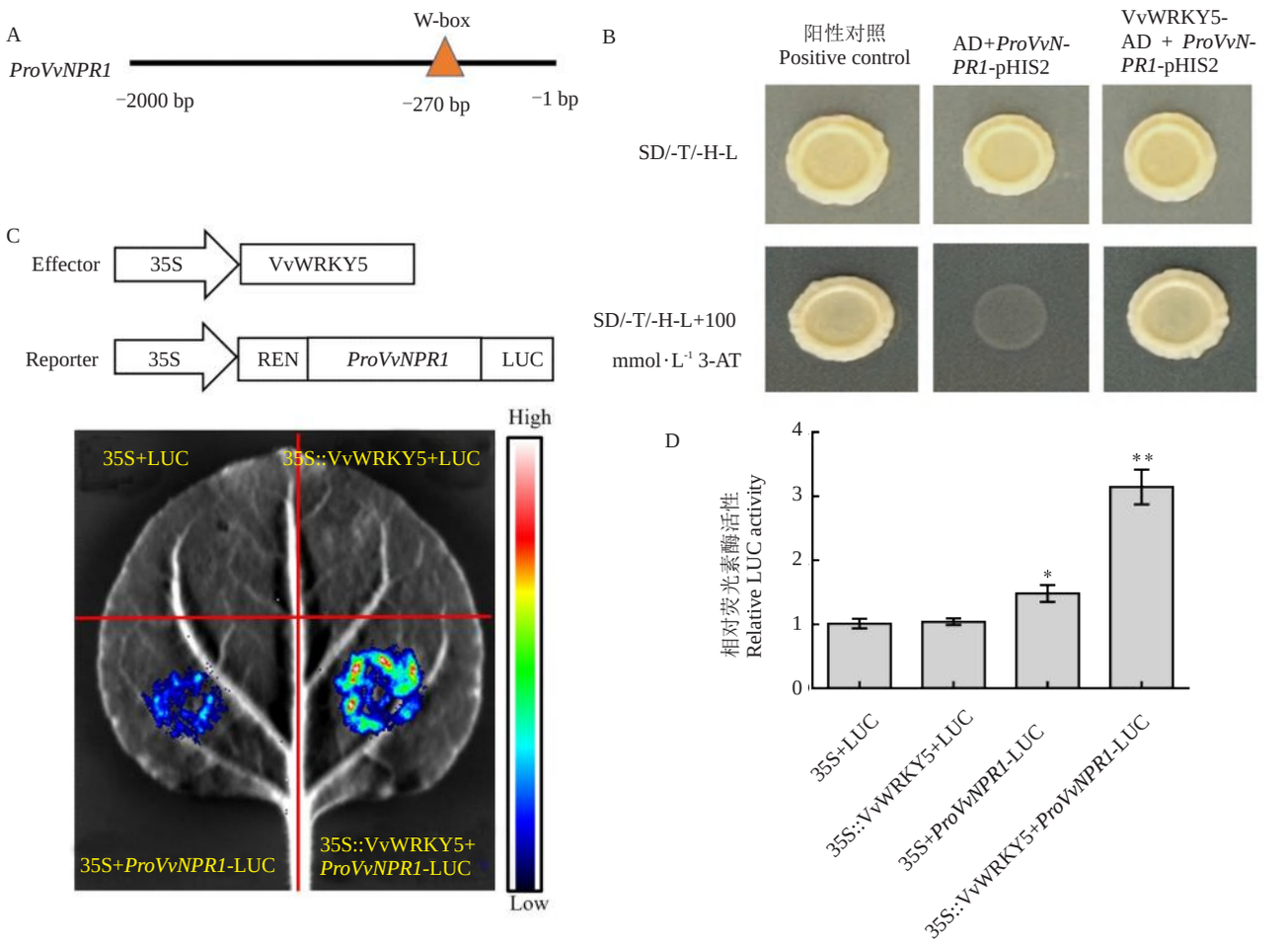
接下来，进行了萤光素酶报告试验以探究VvWRKY5对*VvNPR1*启动子活性的影响。将含有35S::VvWRKY5和*ProVvNPR1*-LUC重组质粒的GV3101农杆菌菌液注射1月龄本氏烟草的叶片，避光培养48 h后利用植物活体成像系统观察烟草叶片的荧光强度。与对照组（35S+LUC、35S::VvWRKY5+LUC、35S+*ProVvNPR1*-LUC）相比，共注射35S::VvWRKY5和*ProVvNPR1*-LUC（35S::VvWRKY5+*ProVvNPR1*-LUC）的烟草叶片荧光信号更为强烈（图7-C）。同时，收集注射孔附近的叶片样品进行LUC和REN活性的测定。结果表明，共注射35S::VvWRKY5和*ProVvNPR1*-LUC的烟草叶片的LUC/REN值明显高于对照组（图7-D）。

### 2.8　VvWRKY5调控葡萄白腐病抗性的作用机制模型

综合分析以上试验数据，笔者提出了VvWRKY5调控葡萄白腐病抗性的作用机制假说。如图8所示，葡萄受白腐病病原菌侵染后，*VvWRKY5*基因的转录水平受胁迫诱导显著上调，进一步通过与植物抗病通路中的核心枢纽基因*VvNPR1*启动子上的W-box顺式作用元件结合，提高*VvNPR1*的转录活性，从而提高了葡萄对白腐病病原菌的防御能力。

## 3　讨　论

在自然环境中，植物的生长发育经常遭受各种生物和非生物胁迫的威胁。为维系生存与繁殖，植物进化出了通过调节自身形态、生理代谢及分子水平的多层次适应性调控网络来实现对环境变化的精准响应与抵御[24-26]。WRKY转录因子是植物中最大的转录因子家族之一，在抗病反应中发挥了重要的作用[27-31]。例如：过表达*CaWRKY27*能够增强辣椒对青枯病的抗性[32]；*FtWRKY15*介导苦荞麦对叶斑病的防御反应[33]；*StWRKY15*在马铃薯抗旱疫病过程中发挥重要作用[34]。本研究发现，葡萄*VvWRKY5*的表达水平受白腐病病原菌的侵染显著上调，过表达*VvWRKY5*基因的葡萄叶片能通过激活防御相关基因的表达，增强对白腐病的抗性，而沉默*VvWRKY5*基因则削弱了葡萄叶片对白腐病的抵抗力。研究结果进一步丰富了WRKY转录因子调控植物胁迫响




A. *VvNPR1* 启动子上的 W-box 位点分析；B. 酵母单杂试验验证 VvWRKY5 可以结合 *VvNPR1* 启动子；C，D. 萤光素酶报告试验验证 VvWRKY5 能够激活 *VvNPR1* 启动子的活性。

A. The W-box motif in*VvNPR1* promoter; B. Yeast one-hybrid assay showing the binding of VvWRKY5 to the *VvNPR1* promoter; C, D. Luciferase reporter assay showing that VvWRKY5 can activate the transcriptional activities of *VvNPR1*.

**图 7 *VvWRKY5* 能与 *VvNPR1* 基因的启动子结合并激活其转录活性**

**Fig. 7 VvWRKY5 binds to the promoter of *VvNPR1* and activates its transcriptional activity**

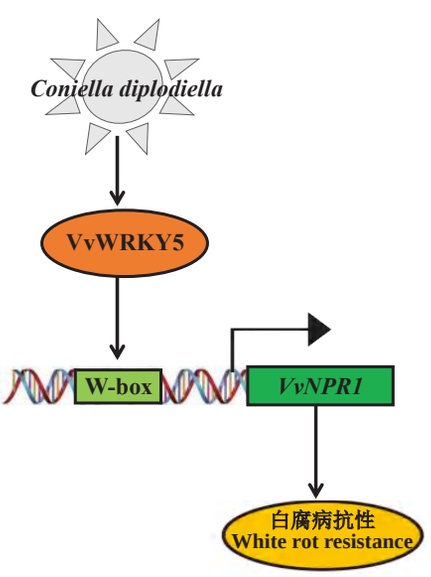



**图 8 葡萄 *VvWRKY5* 在 *VvNPR1* 介导的抗白腐病反应中的作用模式**

**Fig. 8 Proposed model showing the role of VvWRKY5 in *VvNPR1*-mediated white rot resistance in grape**

应的研究，并为通过调控*VvWRKY5*的表达来培育具有更强白腐病抗性的优质葡萄品种提供了理论指导。

有研究表明，WRKY转录因子能够通过与下游靶基因启动子上的W-box位点结合来调节植物的抗病过程[35-36]。*NPR1*作为水杨酸信号途径的核心基因，已有大量研究证明其在植物抗病反应中发挥重要的正调控作用[15，17]。作为水杨酸分子的唯一核心受体，当植物感知病原菌侵染并积累水杨酸信号后，NPR1发生二硫键还原/单体化构象变化，通过核孔复合体转移至细胞核，随后通过与TGA转录因子家族互作形成转录激活复合体，进而特异性地结合并激活下游病程相关基因，最终增强植物的抗病性[37]。本研究通过PlantCARE网站分析发现，在*VvNPR1*基因ATG上游270 bp启动子处包含一个W-box结合元件。酵母单杂交试验发现VvWRKY5能够与*VvNPR1*的启动子直接结合。荧光素酶活性试验表明，VvWRKY5能够提高*VvNPR1*的启动子活性。研究表明VvWRKY5作为上游调控基因促进了*VvNPR1*的表达，进而影响了水杨酸信号通路的转导，提高了葡萄对白腐病的抗性。

目前，利用抗病基因来改良植物的抗病性，已发展成为一种高效且极具潜力的辅助育种手段[38-41]。众多研究表明WRKY类转录因子与植物防御反应密切相关，其不仅数量多、功能多样，而且还参与多种植物激素（如茉莉酸、水杨酸、乙烯等）信号通路[8]。虽然本研究对VvWRKY5抗病机制的探讨尚不十分深入，但笔者证实VvWRKY5能够通过调控水杨酸通路增强葡萄对白腐垫壳孢（*Coniella diplodiella*）的防御反应，表明该基因在葡萄与白腐病病原菌互作过程中可能发挥重要作用。本研究结果不仅有助于阐明激素信号途径介导的葡萄抗白腐病网络机制，也为抗性相关基因的挖掘提供了依据，从而为葡萄抗病育种提供理论参考和新思路。

## 4　结　论

本研究以红地球葡萄为试材，成功克隆获得WRKY转录因子基因*VvWRKY5*，其编码蛋白含WRKY保守结构域和C2H2锌指结构域，与拟南芥AtWRKY22同源性最高，且在葡萄叶片和果实中优势表达。功能验证结果显示，过表达*VvWRKY5*可显著减小葡萄叶片和愈伤组织中白腐病发病面积、减轻病害程度，并上调抗病关键基因*VvNPR1*和*VvPR1*的表达；而沉默*VvWRKY5*则导致相反表型。机制研究进一步证实，VvWRKY5能结合*VvNPR1*启动子并增强其转录活性。综上，VvWRKY5通过靶向激活*VvNPR1*的表达，正向调控葡萄对白腐病的抗性，为葡萄抗病分子机制解析及抗病育种提供了重要基因资源。

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