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·研究论文·

番茄SlWRKY45转录因子在响应低温和干旱胁迫中的功能

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摘要 番茄(*Solanum lycopersicum*)在生长发育过程中常受到低温和干旱等多种非生物胁迫的影响。WRKY转录因子参与调控植物多种非生物胁迫响应过程, 而*SlWRKY45*在番茄非生物胁迫中的功能尚不清楚。基因表达分析发现, 低温、干旱和ABA处理均可显著诱导*SlWRKY45*的表达; 过表达*SlWRKY45*可提高番茄对干旱和低温的耐受性; 在干旱和低温处理下, 过表达株系的光合指标、抗氧化酶活性和脯氨酸(Pro)含量显著高于野生型(WT), 活性氧(ROS)和丙二醛(MDA)含量显著低于WT。转录组数据分析显示, *SlWRKY45*主要通过调控抗氧化酶活性和胁迫响应途径介导番茄对低温胁迫的响应。双荧光素酶报告基因检测发现, SlWRKY45可直接激活*SlPOD1*的表达。酵母双杂交(Y2H)和双分子荧光互补(BiFC)试验结果表明, SlWRKY45与SlWRKY46存在相互作用。综上表明, *SlWRKY45*可能通过直接调控抗氧化酶途径增强转基因番茄的抗逆性, 为番茄的遗传改良提供了重要的候选基因资源。

关键词 番茄, *SlWRKY45*, 干旱胁迫, 低温胁迫, 抗氧化酶

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番茄(*Solanum lycopersicum*)具有丰富的营养价值, 市场需求量大, 在全球各地广泛种植(陈娜等, 2023)。据联合国粮农组织统计, 2022年全球番茄产量达1.86×10^8 t (FAO, https://www.fao.org/faostat/en/#data, 2022)。番茄作为重要的喜温喜水蔬菜作物, 在生产过程中常受到干旱和低温等逆境的影响, 造成种子萌发慢, 发芽率低, 植株生长迟缓以及落花落果等, 严重时导致植株死亡(董舒超等, 2024; 赵来鹏等, 2024)。在受到不同逆境胁迫时, 番茄可通过植株形态、生理代谢及调控机制等途径提高自身的适应性(Li et al., 2024)。大量研究表明, 转录因子能够通过调控特定靶基因的表达, 将感知的逆境信号传递至下游, 从而促使植株对不同逆境做出响应(Baillo et al., 2019)。

WRKY转录因子因其WRKY结构域包含高度保守的“WRKYGQK”位点而得名(Wang et al., 2022), 最初在甘薯(*Ipomoea batatas*)中被发现(Ishiguro and Nakamura, 1994)。目前在水稻(*Oryza sativa*)、拟南芥(*Arabidopsis thaliana*)、棉花(*Gossypium hirsutum*)、番茄、马铃薯(*S. tuberosum*)和茄子(*S. melongena*)等多种植物中鉴定到众多WRKY转录因子, 并进行了相关的功能研究(叶相相等, 2023)。例如, 水稻*OsWRKY24*/*70*/*53*共同参与调控籽粒大小(Tang et al., 2022)。野草莓(*Fragaria vesca*) FvWRKY48通过结合果胶裂解酶基因*FvPLA*的启动子, 控制果实的软化过程(Zhang et al., 2022b)。黄瓜(*Cucumis sativus*) CsWRKY11能与CsNPR1协同调控水杨酸(salicylic acid, SA)诱发的叶片脱绿(Zhang et al., 2024)。柑橘(*Citrus sinensis*) CsWRKY23通过参与SA合成途径、活性氧(reactive oxygen species, ROS)积累和细胞壁强化, 增强果实对青霉菌(*Penicillium digitatum*)的抗性(Wang et al., 2023)。*SlWRKY31*/*46*通过不同途径参与调控番茄对灰霉菌(*Botrytis cinerea*)的抗性(Shu et al., 2021; Huang et

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al., 2022a)。在非生物胁迫响应方面, MdWRKY115通过直接结合*MdRD22*启动子, 提高苹果(*Malus pumila*)对干旱和渗透胁迫的耐受性(Dong et al., 2024)。MsWRKY22可激活*MsWRKY11*的表达, 调控苜蓿(*Medicago sativa*)的抗旱性(Wen et al., 2021)。*TaWRKY31*通过清除ROS、减少气孔开放和调控胁迫相关基因的表达, 最终增强植物的抗旱性(Ge et al., 2024)。*TaWRKY17*通过调节脱落酸(abscisic acid, ABA)/ROS相关基因和胁迫响应基因的表达, 提高自身的抗氧化应激能力, 从而增强转基因拟南芥以及小麦(*Triticum aestivum*)的耐盐性(Yu et al., 2023)。MsWRKY33通过激活*MsERF5*的转录调控ROS的清除, 提高苜蓿的耐盐性(Ma et al., 2023)。*CsWRKY46*通过ABA依赖途径调控冷胁迫应答基因的表达, 从而提高黄瓜的抗寒性(Zhang et al., 2016)。MdWRKY40通过与MdMYB15L结合, 释放*MdCBF2*的表达, 提高苹果的耐寒性(Xu et al., 2023)。EjWRKY7和EjCML19相互协同调控氯化钙介导的枇杷(*Eriobotrya japonica*)果实冷害响应过程(Hou et al., 2023)。

此外, WRKY转录因子的功能往往具有“一因多效”的特点。过表达*OsWRKY45*可同时增强水稻的抗病性和抗旱性(Qiu and Yu, 2009)。*TaWRKY75-A*/*10*同时参与调控小麦对干旱和盐胁迫的耐受性(Wang et al., 2013; Ye et al., 2021)。过表达*GmWRKY12*可通过提高转基因大豆(*Glycine max*)幼苗的脯氨酸(proline, Pro)含量, 降低丙二醛(malondialdehyde, MDA)含量, 增强植株对干旱和盐胁迫的耐受性(Shi et al., 2018)。过表达*OsWRKY11*可提高水稻幼苗的耐旱性和耐高温性(Wu et al., 2009)。异源过表达*ZmWRKY65*可增强转基因拟南芥对病原体攻击以及干旱、盐和低温等多种非生物胁迫的抗性(Huo et al., 2021)。异源过表达*FvWRKY42*可增强转基因拟南芥对白粉病和多种渗透胁迫的抗性以及对ABA的敏感性(Wei et al., 2018)。

已有研究表明, 番茄WRKY转录因子在响应干旱和低温等胁迫过程中发挥重要调控作用。*SlWRKY80*介导的茉莉酸(jasmonic acid, JA)途径通过提高亚精胺含量和维持Na^+/K^+稳态, 正向调节番茄对盐胁迫的抗性(Shang et al., 2024)。*SlWRKY8*/*39*/*52*正向调控番茄对干旱胁迫的抗性(Sun et al., 2015; Gao et al., 2020; Jia et al., 2023)。*SlWRKY81*通过H_2O_2介导的气孔关闭在番茄干旱胁迫响应过程中起负调控作用(Ahammed et al., 2020)。*SlWRKY2*/*6*/*8*/*33*/*45*/*46*/*50*的表达受低温胁迫显著诱导(陈青奇等, 2018); 且过表达*SlWRKY33*/*50*可增强番茄植株的耐冷性(Guo et al., 2022; Wang et al., 2024)。因此, 深入挖掘番茄中低温和干旱等胁迫响应相关的WRKY转录因子, 可为解析该转录因子家族的复杂调控机制及培育优良的抗逆番茄品种提供重要理论依据。

本研究基于实验室前期番茄低温处理的转录组测序(RNA-seq)数据, 进一步通过荧光定量PCR (qRT-PCR)检测, 鉴定出同时受低温和干旱显著诱导的*SlWRKY45*基因。虽然已有研究表明, *SlWRKY45*通过JA合成和信号转导途径负调控番茄对南方根结线虫(*Meloidogyne incognita*)的抗性(Huang et al., 2022b), 但该转录因子在低温和干旱等非生物胁迫中的功能尚未被揭示。我们通过农杆菌介导的番茄遗传转化, 获得*SlWRKY45*的过表达株系。通过对过表达株系在干旱和低温处理下的表型、生理生化指标和RNA-seq分析以及互作蛋白和靶基因筛选, 初步明确了*SlWRKY45*可正向调控番茄的抗旱性和耐低温性。

1 材料与方法

1.1 实验材料及处理

供试材料为番茄品种Micro-Tom (*Solanum lycopersicum* L. cv. ‘Micro-Tom’)。在温度为(26±2)°C, 光周期为16小时光照/8小时黑暗的植物生长室培育野生型和转基因番茄材料。烟草材料为本实验室保存的本氏烟草(*Nicotiana benthamiana* Domin), 在16小时光照(22°C)/8小时黑暗(18°C)的光照培养箱中培育。在不同发育时期分别取番茄的根、茎、叶、花、绿熟期果实、破色期果实和红熟期果实, 迅速置于液氮中, –80°C冰箱保存备用。

待野生型番茄植株长至四叶期, 分别进行ABA (100 μmol·L^{-1}) (处理时间点: 0、3、6、12、24和48小时)、低温(4°C) (处理时间点: 0、6、12、24和48小时)和干旱(20% PEG6000) (处理时间点: 0、3、6、12和24小时)处理。以未做处理作为对照, 在不同时间点分别选取6株幼苗的叶片混合后, 迅速置于液氮中, –80°C冰箱保存备用。

1.2 WRKY系统进化树构建和多序列比对

从番茄Sol Genomics Network (http://solgenomics.net/)、NCBI (http://www.ncbi.nlm.nih.gov/)和TAIR (https://www.arabidopsis.org/)数据库下载SlWRKY45 (Solyc08g067360.3.1)蛋白序列及其同源蛋白序列, 通过DNAMAN 8.0软件对序列进行比对。利用MEGA 5.0软件采用邻接法(neighbor-joining method)构建系统进化树, Bootstrap值设为1 000次。

1.3 RNA提取、反转录及qRT-PCR

使用植物多糖多酚总RNA提取试剂盒(天根, Cat No.DP441)提取番茄不同组织及不同处理样品的RNA。使用StarScript II First-strand cDNA Synthesis Mix With gDNA Remover试剂盒(GenStar, Cat No.A230)对上述RNA样品进行反转录, 合成cDNA。使用Primer 5.0软件设计qRT-PCR的特异性引物(附表1)。使用SYBR qPCR Mix Kit (GenStar, Cat No.A303)和Applied Biosystems StepOnePlus荧光定量仪进行qRT-PCR。以*SlEF1α* (Solyc06g005060)为内参基因, 每个反应设3次重复, 利用$2^{-\Delta\Delta CT}$方法(Livak and Schmittgen, 2001)计算基因的相对表达量, 用*t*-test进行差异显著性分析。

1.4 *SlWRKY45*的克隆

以番茄叶片的cDNA为模板, 利用2× SuperNova PCR Mix (GenStar, Cat No.A064)进行PCR扩增。将扩增产物连至克隆载体(pMD19-T)中, 转化DH5α大肠杆菌感受态细胞, 挑取单菌落进行PCR检测, 并送生工生物公司测序。相关引物序列见附表1。

1.5 酵母自激活实验

从番茄叶片的cDNA中扩增*SlWRKY45*的CDS序列。利用inNova RE III One-Step Cloning Mix (GenStar, Cat No.E104-01)同源重组法将*SlWRKY45*的CDS序列构建到pGBKT7载体的*Eco*RI和*Bam*HI之间, 获得重组质粒pGBKT7-SlWRKY45。相关引物序列见附表1。将pGBKT7空载质粒(阴性对照)、pGBKT7-53+pGADT7-T (阳性对照)和pGBKT7-SlWRKY45分别转入Y2HGold酵母细胞中。挑取单斑进行PCR检测后, 将上述3种阳性单斑稀释100倍, 取4 μL分别点到SD/–Trp、SD/–Trp/–Ade/–His和SD/–Trp/–His/–Ade+X-α-gal培养基上, 30°C倒置培养3–5天。

1.6 *SlWRKY45*的番茄遗传转化和鉴定

利用Gateway™ BP Clonase™ II酶(Invitrogen, Cat No.11789100)将*SlWRKY45*的CDS序列构建到入门载体pDONR207上; 再利用Gateway™ LR Clonase™ II (Invitrogen, Cat No.11791020)将其构建到表达载体pGWB2上, 获得重组质粒pGWB2-*SlWRKY45*。将阳性质粒转化至GV3101农杆菌, 采用农杆菌介导的叶盘法转化番茄品种Micro-Tom (郭静等, 2023)。利用CTAB法提取转基因番茄不同株系的基因组DNA, 以特异性鉴定引物进行PCR扩增, 鉴定阳性转基因株系。相关引物序列见附表1。提取转基因番茄株系的RNA, 利用qRT-PCR检测不同转基因番茄株系中*SlWRKY45*的表达量。

1.7 干旱和低温处理过表达*SlWRKY45*转基因番茄

在相同环境下培育过表达*SlWRKY45*转基因(*SlWRKY45*-OE) T_3代和野生型(WT)番茄, 待幼苗长至四叶期, 分别进行干旱和低温处理。

干旱处理: 挑选长势基本一致的*SlWRKY45*-OE和WT幼苗进行干旱处理, 每组处理9株, 设置3个生物学重复。及时观察植株的生长状况, 并拍照记录表型。

低温处理: 挑选长势基本一致的*SlWRKY45*-OE和WT幼苗进行4°C低温处理, 每组处理9株, 设置3个生物学重复。及时观察植株的生长状况, 并拍照记录表型。

1.8 生理生化指标测定

采集低温处理3天和干旱处理5天的*SlWRKY45*-OE和WT番茄叶片, 分别加入二氨基联苯胺(diaminobenzidine, DAB)和氮蓝四唑(nitrotetrazolium blue chloride, NBT)染色液, 抽真空30分钟; 避光浸染6小时; 用蒸馏水漂洗3次; 浸入组织脱色液中, 80°C水浴30分钟; 用蒸馏水漂洗3次。观察叶片颜色并拍照。

分别采集低温和干旱处理24小时的*SlWRKY45*-OE和WT番茄叶片, 利用过氧化物酶(peroxidase, POD)、超氧化物歧化酶(superoxide, SOD)、丙二醛

(malondialdehyde, MDA)含量和脯氨酸(proline, Pro)含量测定试剂盒(索莱宝)测定POD、SOD活性及MDA和Pro含量。采用*t*-test对不同处理进行差异显著性分析。

利用光合仪(Li-6400)和叶绿素含量测定仪(SPAD-502)分别测定处理前、低温处理3天和干旱处理7天的*SlWRKY45*-OE和WT番茄叶片的净光合速率、气孔导度和叶绿素含量。

分别采集低温处理前和低温处理24小时的*SlWRKY45*-OE和WT番茄叶片, 参照孙红等(2018)的方法, 利用电导率仪(DDBJ-350)测定叶片的相对电导率。

1.9 *SlWRKY45*-OE和WT在低温处理前后的RNA-seq分析

为探究*SlWRKY45*调控低温胁迫响应的分子机制, 我们对*SlWRKY45*-OE (OE#1)和WT低温处理前和低温处理3天的番茄叶片进行取样, 每个样品设置3个生物学重复, 送至上海元莘生物医药科技有限公司完成高通量测序。对测序得到的原始序列信息进行过滤, 得到Clean Data, 并将其比对到番茄基因组的参考基因组序列, 得到Mapped Data, 依据FPKM值计算不同样品组中的差异基因, 筛选标准为$|\log_2(\text{Fold change})|\geq 1$, 多重假设检验校正后$P<0.01$。利用Blast2GO软件和KEGG数据库(http://www.kegg.jp/kegg)分别对差异基因进行GO功能注释和KEGG富集分析(李佩婷等, 2022)。

1.10 双荧光素酶报告基因检测

将*SlWRKY45*的CDS序列、*SlPOD1*和*SlPOD*上游1.5 kb序列分别构建到pGreenII 62-SK和pGreenII 0800-LUC载体中, 获得62SK-SlWRKY45、*SlPOD1*-pro-LUC和*SlPOD* pro-LUC重组载体。相关引物序列见附表1。将重组载体分别转入GV3101 (pSoup-p19)农杆菌, 将*SlPOD1* pro-LUC和*SlPOD* pro-LUC转化菌液分别与62SK (阴性对照)和62SK-SlWRKY45转化菌液按1:1 (v/v)的比例混合后, 注射烟草叶片, 弱光培养2天后, 使用双荧光素酶报告基因检测试剂盒(Beyotime Biotechnology, Shanghai, China)按照操作说明书测定LUC/REN值。

1.11 酵母双杂交

通过同源重组方法将*SlWRKY46*和*SlWRKY45*的CDS序列分别构建到pGBKT7和pGADT7载体*Eco*RI和*Bam*HI之间, 获得重组质粒pGADT7-SlWRKY45和pGBKT7-SlWRKY46。相关引物序列见附表1。将pGBKT7+pGADT7-SlWRKY45 (阴性对照)、pGBKT7-SlWRKY46+pGADT7 (阴性对照)、pGBKT7-53+pGADT7-T (阳性对照)、pGBKT7-SlWRKY46+pGADT7-SlWRKY45分别转入Y2HGold酵母细胞中。挑取单斑进行PCR检测后, 将上述4种阳性单斑分别稀释10、100和1 000倍, 取4 μL分别点到SD/–Trp/–Leu和SD/–Trp/–Leu/–His/–Ade培养基上, 30°C倒置培养3–5天。

1.12 双分子荧光互补

通过同源重组方法将*SlWRKY46*和*SlWRKY45*的CDS序列分别构建到pSm35s-cYFP和pSm35s-nYFP载体中, 获得重组质粒nYFP-SlWRKY45和SlWRKY46-cYFP。相关引物序列见附表1。将构建好的载体转入GV3101农杆菌。分别将不同组合(nYFP-SlWRKY45+cYFP (阴性对照)、nYFP+SlWRKY46-cYFP (阴性对照)和nYFP-SlWRKY45+SlWRKY46-cYFP)的菌液注射烟草叶片, 弱光培养2天后, 在激光共聚焦显微镜(Nikon C2-ER)下观察标记叶片的黄色荧光信号并拍照记录。

2 结果与分析

2.1 *SlWRKY45*的表达分析

对实验室前期的番茄低温处理RNA-seq数据进行分析和筛选, 发现WRKY转录因子家族中的*SlWRKY45*受低温胁迫显著诱导表达(图1A)。为进一步验证转录组数据, 我们通过qRT-PCR分析*SlWRKY45*在低温处理不同时间点的表达特点, 发现该基因的表达呈下降-上升趋势, 尤其在48小时上调最为显著, 上调倍数达434.7倍(图1B)。分析*SlWRKY45*在干旱处理下的表达特点, 发现该基因的表达显著上调, 尤其在24小时上调最为显著, 上调倍数达33.4倍(图1C)。在ABA处理下, *SlWRKY45*的表达显著上调, 尤其在3小时上调最为显著, 上调倍数达31.1倍(图1D)。分析*SlWRKY45*在不同组织中的表达特点, 发现该基因除在茎中表达量低, 在其它组织中的表达量均较高, 特别是在根和破色期果实中的表达量最高(图1E)。

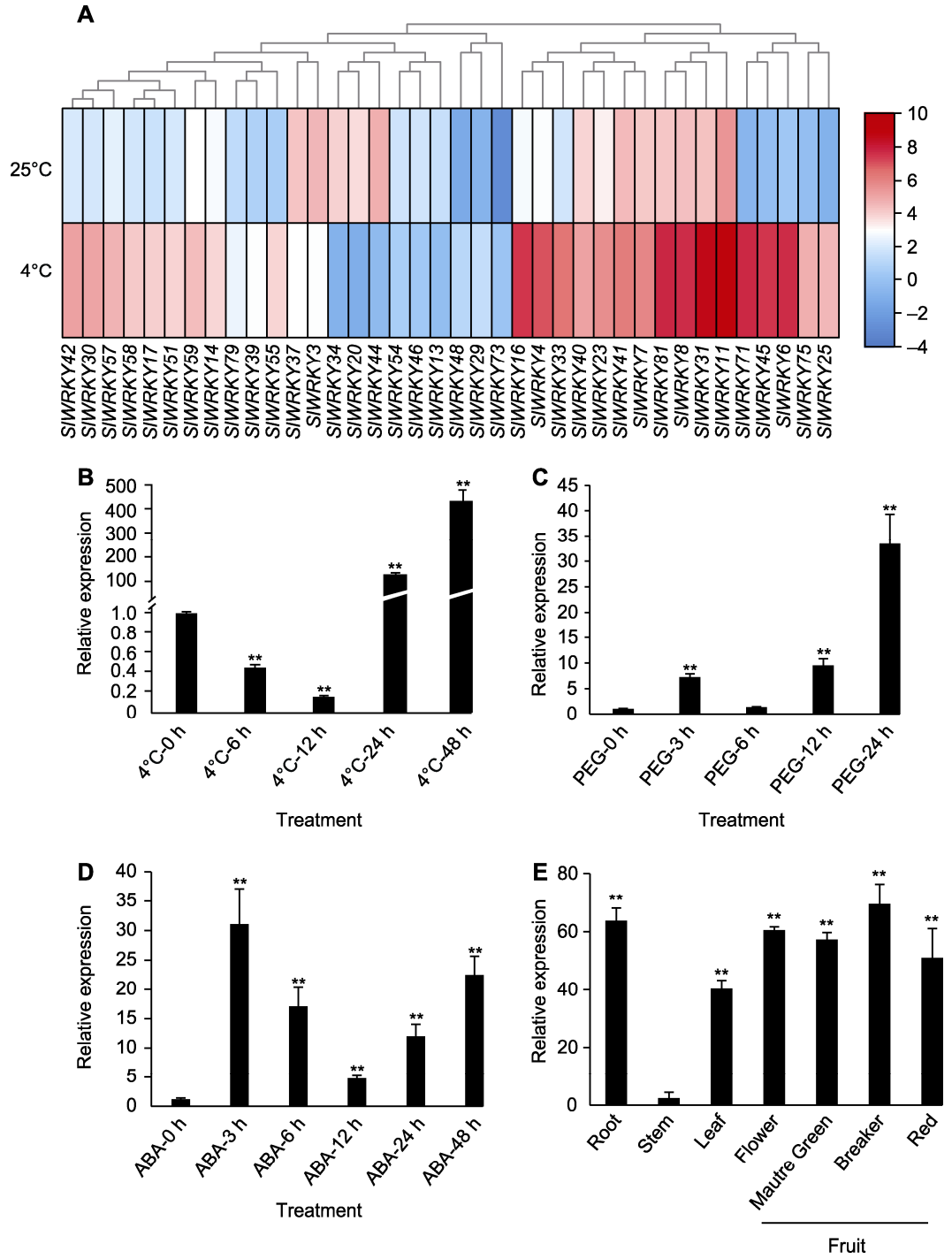


图1 低温处理下*SlWRKYs*的表达热图**(A)**以及*SlWRKY45*在低温**(B)**、干旱**(C)**、脱落酸(ABA) **(D)**处理下和不同组织**(E)**中的表达 **(B), (C), (D)** *SlWRKY45*的相对表达量分别以4°C、ABA和PEG6000处理0小时为对照; **(E)** *SlWRKY45*的相对表达量以茎为对照。** 表示在*P*<0.01水平上差异显著。

Figure 1 Heat map of *SlWRKYs* under low-temperature treatment **(A)** and *SlWRKY45* expression under 4°C **(B)**, drought **(C)**, and abscisic acid (ABA) **(D)** treatments and in different tissues **(E)**
(B), (C), (D) The relative expression levels of *SlWRKY45* at 0 h under treatments of 4°C, ABA and PEG6000 were utilized as controls, respectively; **(E)** The relative expression of *SlWRKY45* in stems served as the control. ** represents significant differences at *P*<0.01.

2.2 *SlWRKY45*的克隆及生物信息学分析

以番茄叶片中提取的RNA为模板合成cDNA, 并以此为模板进行PCR扩增, 获得750 bp左右的单一目的条带(附图1A)。经测序验证, *SlWRKY45*的CDS长度为777 bp, 编码258个氨基酸, 预测分子量和等电点分别为29.3 kDa和5.6。利用SMART网站分析SlWRKY45的蛋白结构, 发现其包含1个WRKY结构域。通过对SlWRKY45和3个拟南芥WRKY转录因子(AtWRKY18、AtWRKY40和AtWRKY60)蛋白序列进行多序列比对, 结果发现SlWRKY45的WRKY结构域中除包含高度保守的“WRKYGQY”核心序列外, 还包含1个C_2H_2类型的锌指结构(附图1B), 表明SlWRKY45属于IIa类型的WRKY转录因子。

对II亚族的44个拟南芥WRKY转录因子以及来源于9种植物IIa亚族的12个WRKY转录因子的蛋白序列构建系统进化树, 经分析发现SlWRKY45序列与CmWRKY19 (冷诱导) (张高原和魏兵强, 2020)、GmWRKY27 (响应干旱和盐胁迫) (Wang et al., 2015)和CsWRKY21 (响应冷胁迫) (Mi et al., 2024)同源性最高, 序列相似度均在50%以上(附图1C), 暗示*SlWRKY45*可能具有类似的调控功能。

2.3 SlWRKY45的转录活性分析

利用酵母系统分析SlWRKY45转录因子的转录活性。将重组质粒pGBKT7-SlWRKY45、pGBKT7空载质粒(阴性对照)和pGBKT7-53+pGADT7-T (阳性对照)转化酵母Y2HGold细胞, 分别点在SD/–Trp、SD/–Trp/–His/–Ade和SD/–Trp/–His/–Ade+X-α-gal培养基上进行检测。结果发现, 在SD/–Trp培养基上, pGBKT7-SlWRKY45、阴性对照和阳性对照均能正常生长; 在SD/–Trp/–His/–Ade和SD/–Trp/–His/–Ade+X-α-gal培养基上, pGBKT7-SlWRKY45和阳性对照正常生长且变蓝, 阴性对照不能正常生长(图2)。该结果表明, SlWRKY45转录因子具有转录激活活性。

2.4 *SlWRKY45*-OE转基因番茄在干旱胁迫下的表型及生理生化指标

为探究*SlWRKY45*在番茄胁迫响应过程中的功能, 构建*SlWRKY45*的过表达载体并遗传转化番茄, 利用PCR鉴定到4个过表达*SlWRKY45*的转基因番茄株系(#1、#2、#5和#7) (附图2A)。利用qRT-PCR对上述4个过表达株系的*SlWRKY45*表达量进行分析, 发现*SlWRKY45*在#1、#2、#5和#7中的表达量均显著高于野生型(WT) (附图2B), 选取#1和#2两个株系进行后续相关实验。

对*SlWRKY45*-OE转基因和WT番茄幼苗进行干旱处理。结果发现干旱处理前, *SlWRKY45*-OE转基因和WT植株表型无明显差异; 在干旱处理7天、10天和15天后, *SlWRKY45*-OE转基因植株叶片的萎蔫程度较WT均明显减轻(图3A)。

为了分析过表达*SlWRKY45*是否影响番茄的光合能力, 检测干旱处理前后的叶绿素含量、光合速率和气孔导度。结果发现, 干旱处理前, *SlWRKY45*-OE转基因和WT间无显著差异; 干旱处理后, *SlWRKY45*-OE转基因番茄的叶绿素含量、光合速率和气孔导度均显著高于WT (图3B)。表明过表达*SlWRKY45*对于维持干旱胁迫下番茄的光合能力具有重要作用。

为了检测过表达*SlWRKY45*对番茄ROS积累及

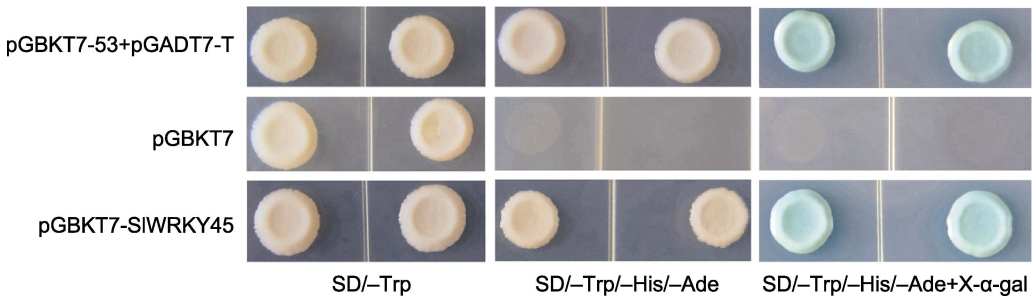


图2 SlWRKY45的转录自激活
pGBKT7-53+pGADT7-T和pGBKT7空载质粒分别为阳性对照和阴性对照。

Figure 2 Transcriptional self-activation of SlWRKY45
pGBKT7-53+pGADT7-T and the empty vector pGBKT7 represent the positive control and negative control, respectively.

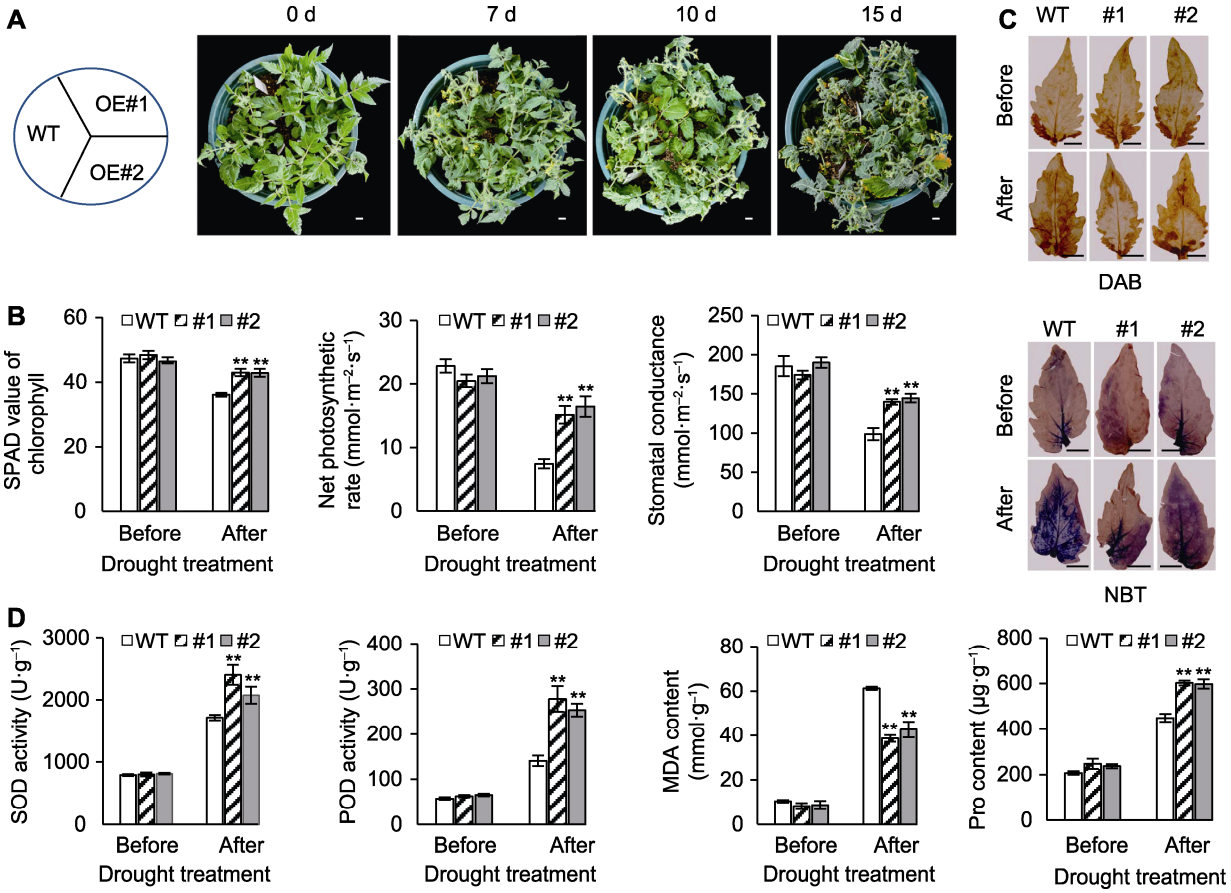


图3 干旱处理下*SlWRKY45*-OE转基因和WT番茄植株的表型**(A)**、光合指标**(B)**、DAB和NBT染色**(C)**及生理生化指标**(D)**

(B), (D) **表示在*P*<0.01水平上差异显著。DAB: 二氨基联苯胺; NBT: 氮蓝四唑; WT: 野生型; SOD: 超氧化物歧化酶; POD: 过氧化物酶; MDA: 丙二醛; Pro: 脯氨酸. #1和#2为不同的转基因株系。Bars=1 cm

Figure 3 Phenotype **(A)**, photosynthesis indicators **(B)**, DAB, and NBT staining **(C)** and physiological indicators **(D)** of *SlWRKY45*-OE transgenic and WT tomato plants under drought treatment

(B), (D) ** represent significant differences at *P*<0.01. DAB: Diaminobenzidine; NBT: Nitrotetrazolium blue chloride; WT: Wild type; SOD: Superoxide; POD: Peroxidase; MDA: Malondialdehyde; Pro: Proline. #1 and #2 are different transgenic lines. Bars=1 cm

清除的影响，对干旱处理前后的*SlWRKY45*-OE转基因植株和WT的叶片分别进行DAB和NBT染色。结果发现，干旱处理前，二者叶片染色无明显差异；干旱处理后，*SlWRKY45*-OE转基因植株和WT的叶片染色均加深，但*SlWRKY45*-OE比WT染色较浅(图3C)。测定*SlWRKY45*-OE转基因植株和WT叶片的SOD和POD活性以及MDA和Pro含量，发现干旱处理前，二者无显著差异；干旱处理后，*SlWRKY45*-OE转基因植株的SOD活性、POD活性和Pro含量显著高于WT，MDA含量显著低于WT (图3D)。干旱胁迫下，*SlWRKY45*-OE转基因植株较WT的细胞氧化损伤更轻，表明其抗旱性更强。

2.5 *SlWRKY45*-OE转基因番茄在低温胁迫下的表型及生理生化指标

对*SlWRKY45*-OE转基因和WT番茄幼苗进行低温处理后发现，常温下*SlWRKY45*-OE转基因和WT植株无明显差异；在低温处理5天后，*SlWRKY45*-OE转基因和WT的植株叶片均发生了卷曲萎蔫，但*SlWRKY45*-OE转基因植株叶片萎蔫程度较WT轻(图4A)。

检测叶绿素含量和光合速率，发现常温下，二者无显著差异；低温处理下，*SlWRKY45*-OE转基因植株叶片的叶绿素含量和光合速率显著高于WT (图4B)。表明过表达*SlWRKY45*对于维持番茄在低温胁迫下的光合能力具有重要作用。

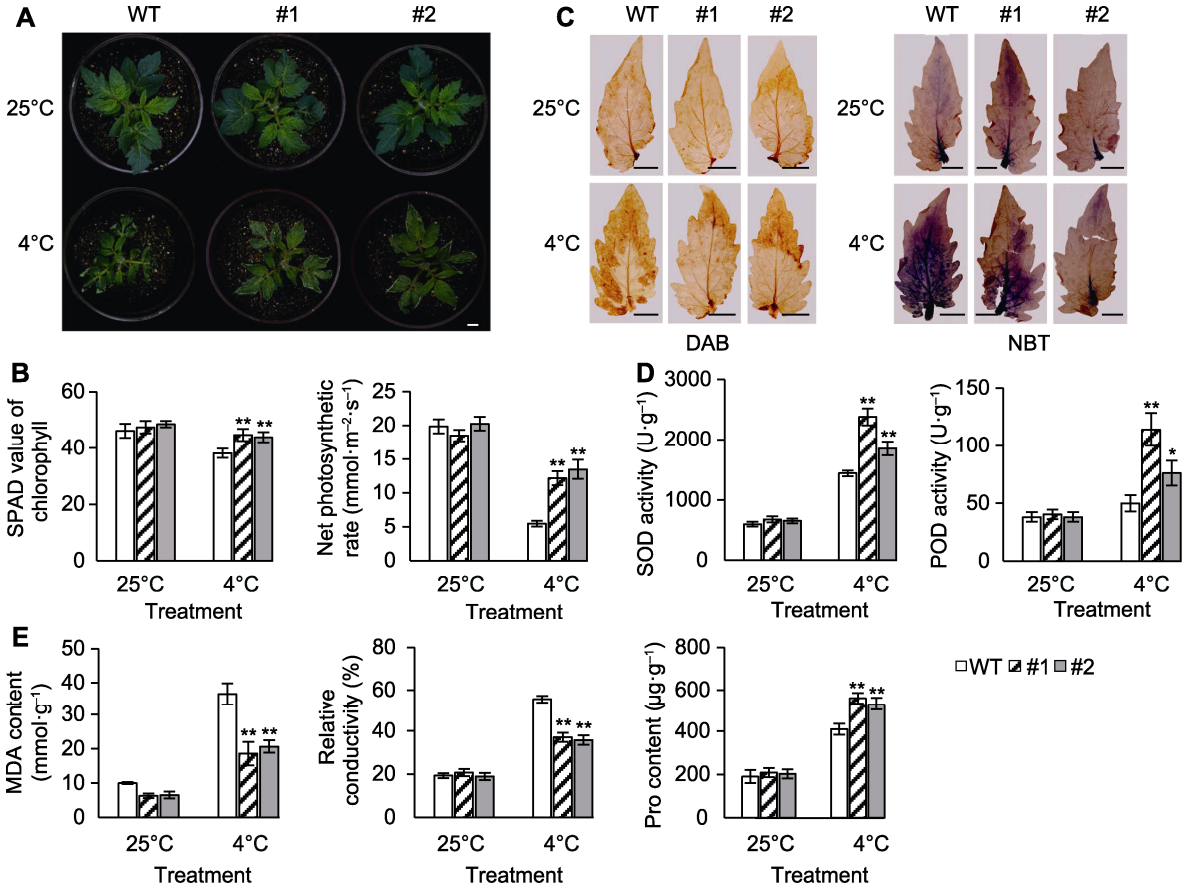


图4 *SlWRKY45*-OE转基因和WT番茄植株在4°C处理下的表型**(A)**、光合指标**(B)**、DAB和NBT染色**(C)**及生理指标**(D), (E)**
(B)–(E) *和**分别表示在*P*<0.05和*P*<0.01水平上差异显著。WT: 野生型。DAB、NBT、SOD、POD、MDA和Pro同图3。#1、#2为不同的转基因株系。Bars=1 cm

Figure 4 Phenotype **(A)**, photosynthesis indicators **(B)**, DAB, and NBT staining **(C)**, and physiological indicators **(D), (E)** of *SlWRKY45*-OE transgenic and WT tomato plants under 4°C treatment
(B)–(E) * and ** represent significant differences at *P*<0.05 and *P*<0.01, respectively. WT: Wild type. DAB, NBT, SOD, POD, MDA, and Pro are the same as shown in Figure 3. #1 and #2 are different transgenic lines. Bars=1 cm

对常温和低温下*SlWRKY45*-OE转基因植株和WT的叶片分别进行DAB和NBT染色。结果发现，常温下二者染色情况无明显差异；低温处理下，*SlWRKY45*-OE转基因植株和WT的叶片染色均加深，但*SlWRKY45*-OE较WT染色浅(图4C)。测定*SlWRKY45*-OE和WT叶片的SOD和POD活性，发现常温下二者的SOD和POD活性无显著差异；低温处理下，*SlWRKY45*-OE的SOD和POD活性显著高于WT (图4D)；检测*SlWRKY45*-OE和WT叶片的MDA含量、相对电导率和Pro含量，发现常温下二者无显著差异；低温处理下，*SlWRKY45*-OE的MDA含量和相对电导率均显著低于WT, Pro含量显著高于WT (图4E)。综上表明，低温处理下，*SlWRKY45*-OE转基因植株较WT的细胞氧化损伤减轻，番茄植株的耐低温性增强。

2.6 *SlWRKY45*-OE转基因番茄在低温胁迫下的RNA-seq分析

为阐明*SlWRKY45*调控番茄低温胁迫的分子机制，我们对低温处理前后的*SlWRKY45*-OE (OE#1)和WT进行RNA-seq。分析RNA-seq数据，发现常温下WT25 vs OE25比较组的差异表达基因(differentially expressed genes, DGEs)共282个，其中190个基因上调, 92个基因下调；低温处理下, WT4 vs OE4比较组的DGEs共826个，其中257个基因上调，569个基因下调(图5A)。与WT25 vs OE25相比, WT4 vs OE4比较组的DGEs包括*AP2* (15个)、*WRKY* (7个)、*MYB* (5

个)和*NAC* (4个)等转录因子以及*POD* (12个)、*SOD* (1个)和*GST* (4个)等抗氧化酶基因。

对DGEs进行GO功能注释分析，发现WT25 vs OE25和WT4 vs OE4比较组的DGEs在调控氧化还原酶活性方面均有较多基因富集，且WT4 vs OE4相较于WT25 vs OE25在胁迫响应过程中基因富集更显著(图5B)。KEGG富集分析表明, DGEs主要富集在次生代谢物的生物合成(ko01110)、植物激素信号转导(ko04075)以及代谢途径(ko01100)等；值得注意的是，抗氧化酶中的过氧化物酶途径(ko04146)仅在

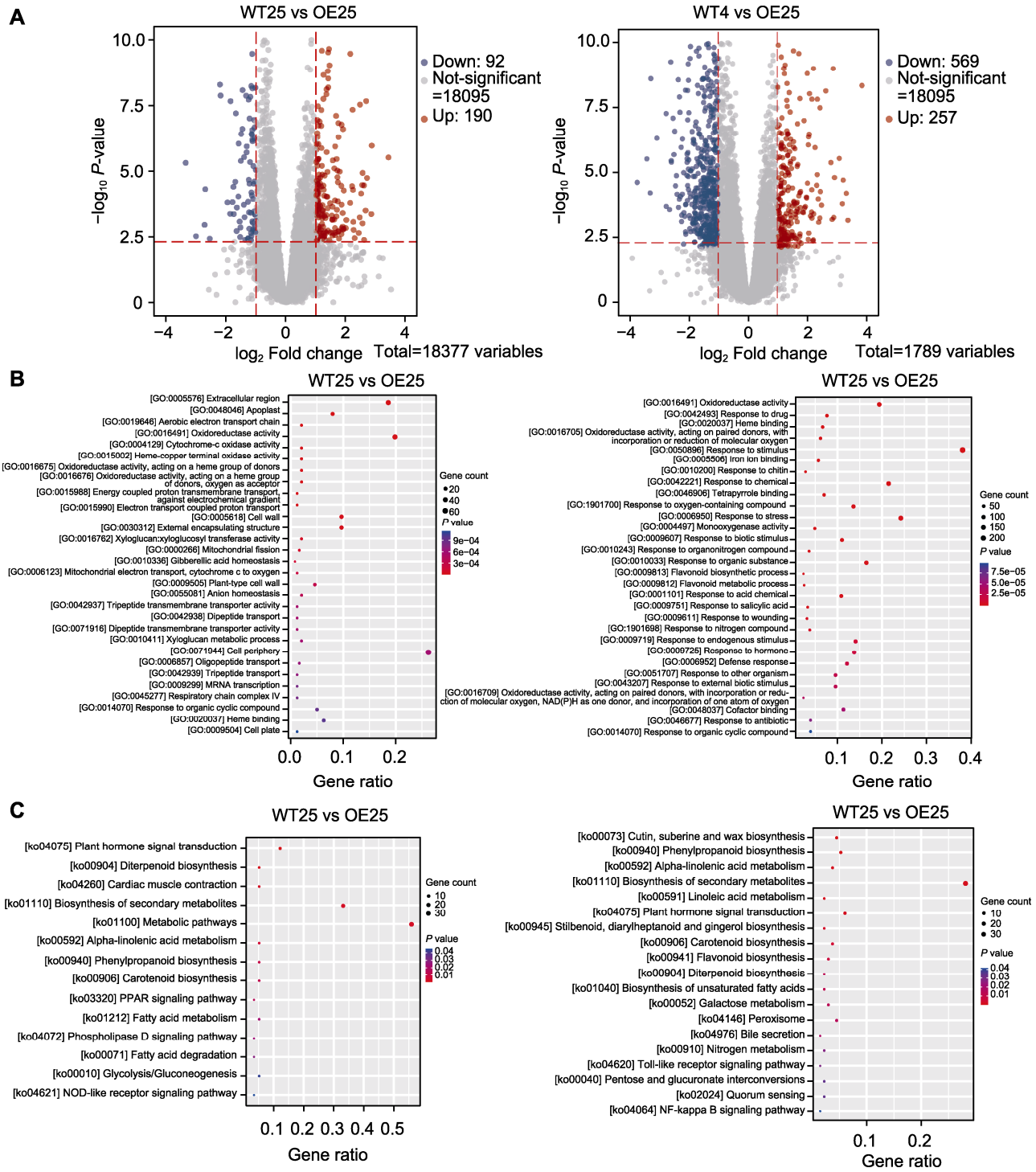


图5
Figure 5

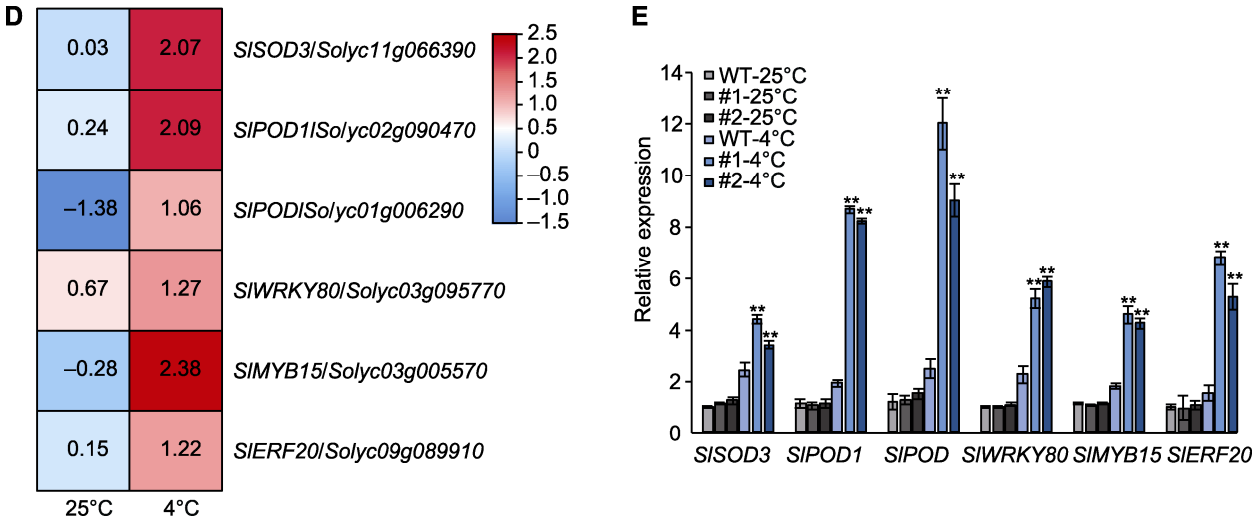


图5 *SlWRKY45*-OE转基因番茄和WT在低温处理前后的RNA-seq分析

(A) 差异表达基因(DEGs)火山图(红色、蓝色和灰色分别代表上调、下调和无差异表达基因); **(B)** GO聚类(红色星号和线条标出的为显著富集的通路); **(C)** KEGG富集分析(红色星号和线条标出的为显著富集的通路); **(D)** 挑选的6个DEGs的表达热图; **(E)** 6个DEGs的相对表达量(以常温下WT为对照。**表示在*P*<0.01水平上差异显著。WT: 野生型。#1和#2为不同的转基因株系)

Figure 5 RNA-seq analysis of *SlWRKY45*-OE transgenic tomato and WT plants before and after low-temperature treatment

(A) Volcano diagram of DEGs (red, blue and gray represent upregulated, downregulated and undifferentially expressed genes, respectively); **(B)** GO cluster (the red asterisks and lines indicate significantly enriched pathways); **(C)** KEGG enrichment (the red asterisks and lines indicate significantly enriched pathways); **(D)** The heatmap of 6 selected DEGs; **(E)** The relative expression of the 6 selected DEGs (the relative expression of WT under normal temperature was used as control. ** represent significant differences at *P*<0.01. WT: Wild type. #1 and #2 are different transgenic lines)

WT4 vs OE4中有富集(图5C)。上述结果表明, *SlWRKY45*可能通过调控抗氧化酶、植物激素信号及次生代谢合成等过程提高番茄的耐低温性。

从WT4 vs OE4比较组的DGEs中选取3个抗氧化酶基因(*SlSOD3* (Solyc11g066390)、*SlPOD1* (Solyc02g090470)和*SlPOD* (Solyc01g006290))以及3个已报道与低温胁迫响应有关的转录因子基因(*SlWRKY80* (Solyc03g095770) (王艺璇等, 2021)、*SlMYB15* (Solyc03g005570) (Zhang et al., 2022a)以及*SlERF20* (Solyc09g089910) (Sharma et al., 2010))进行qRT-PCR检测。结果发现, 常温下上述6个基因在*SlWRKY45*-OE转基因植株与WT间无显著差异; 低温处理下, 这些基因的表达显著上调, 且在*SlWRKY45*-OE中的表达量显著高于WT (图5E), 与RNA-seq结果一致(图5D)。上述结果表明, *SlWRKY45*提高番茄的耐低温性可能与调控抗氧化酶基因及低温胁迫相关转录因子的表达有关。

2.7 SlWRKY45调控番茄耐低温性的靶基因

上述研究发现, *SlWRKY45*调控番茄的耐低温性可能与抗氧化酶途径密切相关(图4)。为了明确*SlWRKY45*是否直接参与调控抗氧化酶途径, 对上述3个差异表达的抗氧化酶基因(*SlSOD3*、*SlPOD1*和*SlPOD*)上游1.5 kb启动子序列的顺式作用元件进行了预测。结果显示, *SlPOD1*和*SlPOD*启动子区分别含有3个WRKY转录因子结合元件W-box-like和W-box (图6A)。选取这2个基因作为*SlWRKY45*可能直接调控的靶基因。分别将*SlWRKY45*的CDS序列及*SlPOD1*和*SlPOD*的1.5 kb启动子序列构建到pGreenII 62-SK和pGreenII 0800-LUC载体中(图6B), 进行双荧光素酶报告基因检测。结果发现, 与62SK+*SlPOD1* pro (对照组)相比, 62SK-SlWRKY45+*SlPOD1* pro的LUC/REN显著升高; 62SK+*SlPOD* pro (对照组)与62SK-SlWRKY45+*SlPOD* pro的LUC/REN无显著差异(图6C)。该结果表明, SlWRKY45可能通过直接激活*SlPOD1*的表达提高番茄的耐低温性。

2.8 SlWRKY45互作蛋白筛选

为探明SlWRKY45的互作蛋白, 我们选择已有报道受低温显著诱导(陈青奇等, 2018), 且实验室前期研究

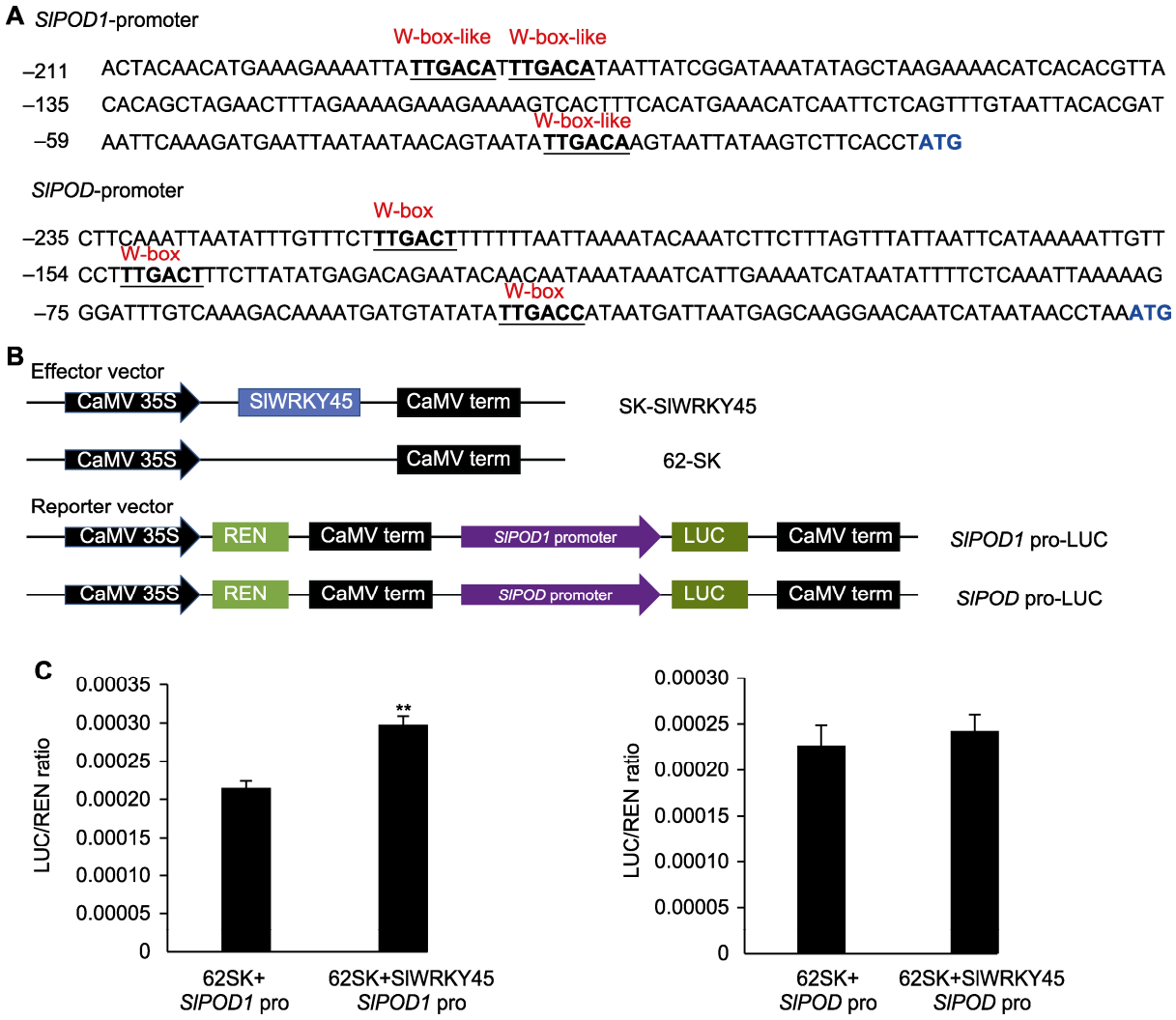


图6 双荧光素酶报告基因检测分析SlWRKY45对*SlPOD1*和*SlPOD*启动子的激活作用

(A) *SlPOD1*和*SlPOD*启动子序列分析(加粗下划线表示W-box-like和W-box元件); **(B)** 报告基因和效应基因的载体示意图; **(C)** LUC/REN分析(以62SK+*SlPOD1* pro和62SK+*SlPOD* pro为对照组。**表示在*P*<0.01水平上差异显著)

Figure 6 Dual-luciferase reporter assays analysis of SlWRKY45 on *SlPOD1* and *SlPOD* promoters activation

(A) Sequence analysis of the *SlPOD1* and *SlPOD* promoters (the bold underlines represent W-box-like and W-box elements); **(B)** Schematic representation of reporter and effector vectors; **(C)** LUC/REN analysis (62SK+*SlPOD1* pro and 62SK+*SlPOD* pro are the control groups. ** represents significant differences at *P*<0.01)

发现在低温胁迫中起正调控作用的SlWRKY46 (未发表数据)，进行二者相互作用分析。在酵母双杂交(yeast two-hybrid, Y2H)实验中，依据前期研究发现的SlWRKY46在酵母系统中不具有自激活活性，将重组质粒pGBKT7-53+pGADT7-T (阳性对照)、pGBKT7+pGADT7-SlWRKY45 (阴性对照)、pGBKT7-SlWRKY46+pGADT7 (阴性对照)、pGBKT7-SlWRKY46+pGADT7-SlWRKY45分别转入Y2HGold酵母细胞中，点在SD/–Trp/–Leu/–His/–Ade培养基上。观察酵母菌落生长情况，发现在SD/–Trp/–Leu培养基上，上述组合均能正常生长；在SD/–Trp/–Leu/–His/–Ade培养基上，pGBKT7-SlWRKY46+pGADT7-SlWRKY45和阳性对照能正常生长，阴性对照无法生长(图7A)。利用双分子荧光互补(bimolecular fluorescence complementation, BiFC)进一步验证SlWRKY45与SlWRKY46的相互作用。以nYFP-SlWRKY45+cYFP和nYFP+SlWRKY46-cYFP为阴性对照，nYFP-SlWRKY45+SlWRKY46-

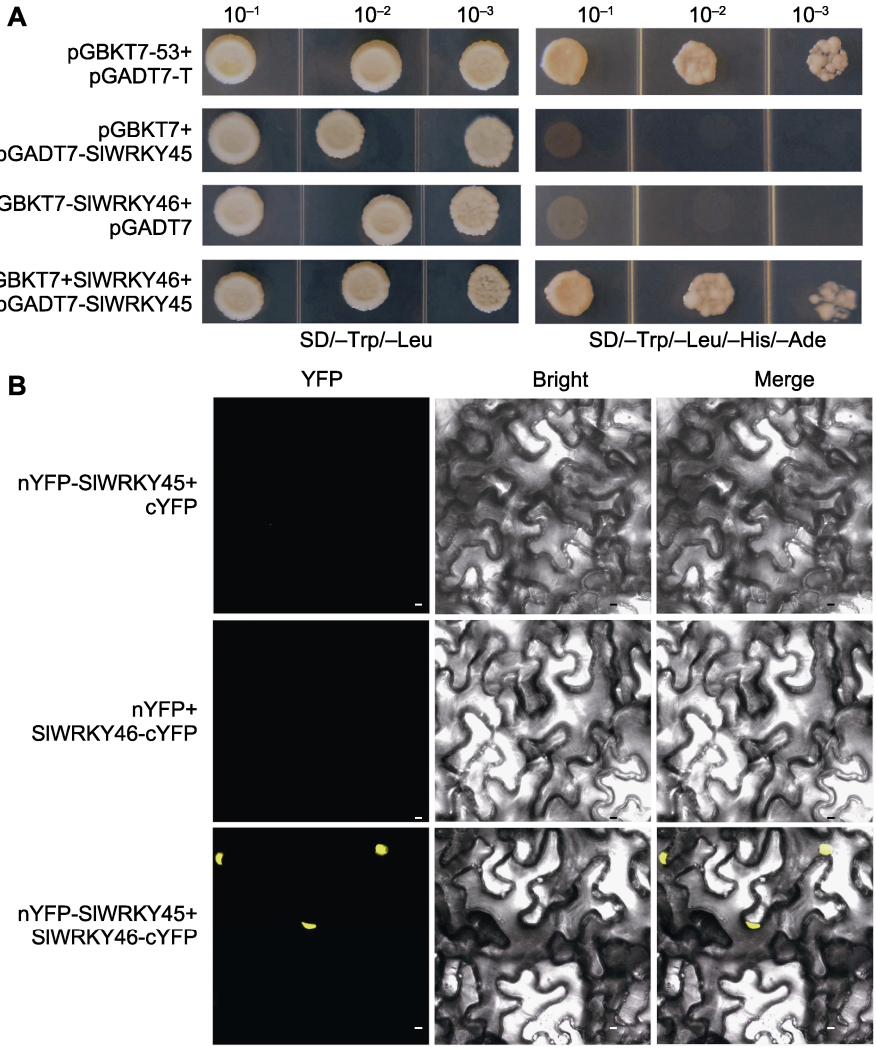


图7 酵母双杂交(Y2H) **(A)**和双分子荧光互补(BiFC) **(B)**检测SlWRKY45与SlWRKY46的相互作用

(A) pGBKT7-53+pGADT7-T为阳性对照, pGBKT7+pGADT7-SlWRKY45和pGBKT7-SlWRKY46+pGADT7为阴性对照; **(B)** 图片从左到右依次为黄色荧光信号、明场和叠加, nYFP-SlWRKY45+cYFP和nYFP+SlWRKY46-cYFP为阴性对照。Bars=20 μm

Figure 7 Interaction of SlWRKY45 and SlWRKY46 via yeast two-hybrid (Y2H) **(A)** and bimolecular fluorescence complementation (BiFC) **(B)**

(A) pGBKT7-53+pGADT7-T represents a positive control, pGBKT7+pGADT7-SlWRKY45 and pGBKT7-SlWRKY46+pGADT7 represent negative controls; **(B)** From left to right, the yellow fluorescent signal, bright field image and merge image are shown, nYFP-SlWRKY45+cYFP and nYFP+SlWRKY46-cYFP represent negative controls. Bars=20 μm

cYFP为实验组，分别注射烟草叶片。结果发现，nYFP-SlWRKY45+SlWRKY46-cYFP在细胞核中观察到明亮的黄色荧光信号，而在阴性对照中检测不到荧光信号(图7B)。上述结果表明SlWRKY45与SlWRKY46存在相互作用。

3 讨论与结论

干旱、低温和盐碱等环境胁迫严重影响植物的生长发育。WRKY在多种逆境胁迫响应过程中具有重要调控作用(Li et al., 2020)。越来越多的研究表明，WRKY转录因子可能在多个非生物胁迫过程中发挥关键调控作用。例如，大豆GmWRKY27通过与GmMYB174互作，抑制*GmNAC29*的表达，提高大豆毛状根的耐旱和耐盐性(Wang et al., 2015)。在拟南芥中异源过表达*FvWRKY42*促进干旱和盐胁迫下的气孔关闭，同时增强植株对白粉病的抗性(Wei et

al., 2018)。过表达*MdWRKY30*可增强转基因拟南芥和苹果愈伤组织对盐胁迫和渗透胁迫的耐受性(Dong et al., 2020)。过表达*SlWRKY23*可提高番茄植株抗*Pst* DC3000和盐胁迫的能力(孙晓春等, 2014)。番茄作为重要的世界性蔬菜作物, 在其生长发育过程中常受到低温和干旱等非生物胁迫的影响(Liang et al., 2022)。结合本实验室前期低温处理番茄的转录组数据以及低温、干旱和ABA处理下的qRT-PCR分析, 我们筛选到受低温和干旱胁迫显著诱导的*SlWRKY45*(图1), 提示*SlWRKY45*可能参与调控番茄的多种非生物胁迫响应。通过对*SlWRKY45*进行分析, 发现该转录因子属于WRKY转录因子家族的IIa亚族(附图1B); 与甜瓜(*C. melo*)中受低温显著诱导的*CmWRKY19* (张高原和魏兵强, 2020)、正调控大豆干旱和盐胁迫的*GmWRKY27* (Wang et al., 2015)以及通过ABA介导调控茶树(*Camellia sinensis*)低温胁迫的*CsWRKY21* (Mi et al., 2024)编码蛋白的序列相似度较高(附图1C), 进一步表明*SlWRKY45*可能在干旱和低温等非生物胁迫响应过程中发挥调控作用。

植物通过改变细胞形态、生理代谢和基因调控等机制抵御非生物胁迫(Baillo et al., 2019; 王芳等, 2019)。前人的研究表明, WRKY转录因子通过清除ROS和渗透调节等机制调控植株抗逆性。*SlWRKY50*通过JA合成途径增强细胞的抗氧化酶活性, 从而提高番茄的耐冷性(Wang et al., 2024)。*SlWRKY52*通过积累渗透调节物质、增强抗氧化酶活性及促进ABA的生物合成提高番茄的抗旱性(Jia et al., 2023)。*SlWRKY81*通过抑制H_2O_2的积累抑制气孔关闭, 从而减弱番茄的抗旱性(Ahammed et al., 2020)。*SbWRKY30*通过提高SOD、POD和CAT的活性减少叶片中ROS的积累, 提高转基因水稻和拟南芥的抗旱性(Yang et al., 2020)。*VvWRKY28*通过影响MDA和Pro等的含量提高葡萄(*Vitis vinifera*)植株的耐冷性(Liu et al., 2022)。本研究通过观察*SlWRKY45*-OE转基因植株和WT在干旱和低温处理下的表型, 发现*SlWRKY45*-OE植株的抗旱和耐低温性增强(图3A, 图4A)。检测光合速率和叶绿素含量等光合指标以及ROS积累、抗氧化酶活性、MDA和Pro的含量, 发现胁迫处理下, *SlWRKY45*-OE转基因植株的ROS积累和MDA含量显著低于WT, 光合指标、SOD活性、POD活性及Pro含量显著高于WT (图3, 图4)。上述结果表明, *SlWRKY45*可能通过提高抗氧化酶活性减轻细胞的氧化损伤, 同时提高植株的渗透调节能力, 维持胁迫下的光合作用, 最终增强番茄植株的抗旱性和耐低温性。

为了探究*SlWRKY45*在番茄低温胁迫响应中的调控机制, 我们对低温处理前后*SlWRKY45*-OE转基因植株和WT进行DEGs、GO和KEGG富集分析, 发现相较于低温处理前, 低温处理后WT4 vs OE4的DEGs数量多, 且多在胁迫响应和过氧化酶途径富集(图5)。该结果进一步验证了生理生化指标的检测结果, 表明*SlWRKY45*提高番茄的耐低温性与抗氧化酶(特别是过氧化物酶)清除ROS途径紧密相关。WRKY转录因子可通过与靶基因启动子区的顺式作用元件W-box (TTGACC/T)和W-box-like (TTGACA)等特异性结合, 调控下游基因的表达(Ye et al., 2021)。为探究*SlWRKY45*是否直接调控抗氧化酶基因的表达, 通过启动序列分析和双荧光素酶报告基因检测, 发现SlWRKY45可激活*SlPOD1*的表达(图6B, C), 表明SlWRKY45可能通过直接靶向调控抗氧化酶基因的表达, 提高番茄的耐低温性。

已有研究表明, WRKY-WRKY存在广泛的相互作用, 共同行使相关的生物学功能。拟南芥中IIa亚族的AtWRKY18/40/60通过N端的亮氨酸拉链区段彼此互作, 以复杂的调控形式参与植株对病原侵染的抵抗、ABA信号途径和非生物胁迫响应(Xu et al., 2006; Chen et al., 2010)。水稻OsWRKY71与OsWRKY51互作, 协同调控ABA和赤霉素介导的种子萌发过程(Chen et al., 2010)。番茄SlWRKY30与SlWRKY81互作, 共同促进*SlPR-STH2*的表达, 提高番茄对青枯病(*Ralstonia solanacearum*)的抗性(Dang et al., 2023)。实验室前期通过对*SlWRKY46*的功能研究, 发现过表达该基因可提高番茄的耐冷性(数据未提供)。SlWRKY46与SlWRKY45同属于IIa亚家族, 均正调控番茄的低温胁迫响应, 且均与抗氧化酶清除ROS途径相关。本研究通过Y2H和BiFC实验, 发现SlWRKY46与SlWRKY45存在相互作用(图7)。由此我们推测SlWRKY45通过与SlWRKY46互作, 协同调控抗氧化酶对ROS的清除, 从而增强番茄的耐低温性。

本研究通过表达分析, 从番茄WRKY转录因子家族中筛选到受低温、干旱和ABA显著诱导表达的*SlWRKY45*。对过表达*SlWRKY45*转基因番茄株系在

干旱和低温处理下的表型和生理生化指标分析表明, 该基因正向调控番茄的抗旱和耐低温性。低温胁迫下的RNA-seq分析、双荧光素酶报告基因检测和互作分析显示, SlWRKY45与SlWRKY46互作, 可能通过直接调控*SlPOD1*的表达参与调控番茄的耐低温性, 研究结果可为深入解析WRKY转录因子家族基因的复杂调控机制提供依据。

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樊蓓: 完成实验, 分析数据, 撰写论文初稿; 任敏、王延峰和党峰峰: 实验技术指导, 协助分析数据; 陈国梁和程国亭: 提供实验设备和技术支持; 杨金雨: 协助完成实验; 孙会茹: 构思设计实验, 修改论文。

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Functions of SlWRKY45 in Response to Low-temperature and Drought Stress in Tomato

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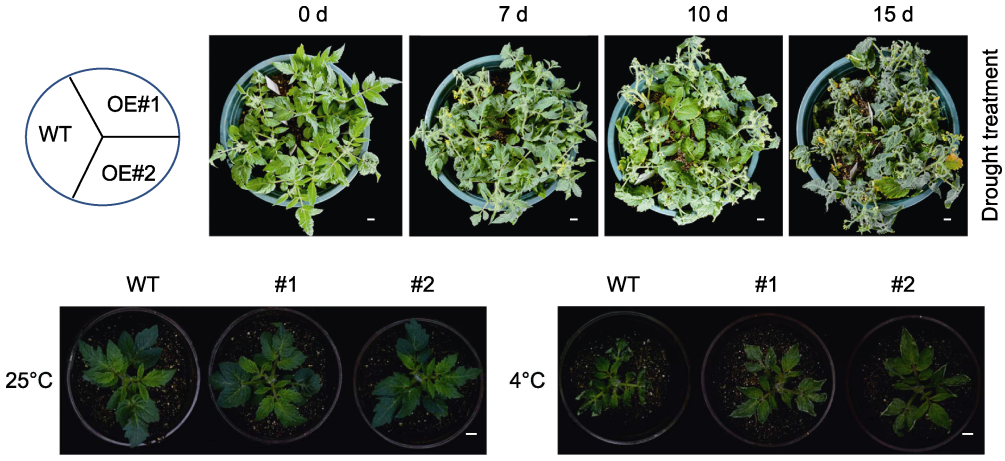
INTRODUCTION: Tomato (*Solanum lycopersicum*), a significant warm-season and water-dependent vegetable crop, is extensively cultivated worldwide. Whether grown in open fields or protected environments, tomatoes frequently encounter various environmental stresses, including drought and low temperatures, which significantly impact their yield and quality. Transcription factors play a pivotal role in plant stress responses by modulating the expression of specific target genes, thereby transmitting perceived stress signals downstream. WRKY transcription factors in tomatoes are known to regulate responses to multiple abiotic stresses. However, the specific role of the tomato SlWRKY45 in abiotic stress responses remains unclear.

RATIONALE: Studies have demonstrated that WRKY transcription factors play a crucial regulatory role in plant responses to abiotic stress. As an important economic vegetable crop, tomato is susceptible to various environmental stresses during its growth and development. By genetically overexpressing *SlWRKY45* in tomato and investigating its function under low-temperature and drought stress conditions, the findings can provide a theoretical foundation for understanding the complex regulatory mechanisms of WRKY transcription factors. Additionally, this research offers valuable candidate genes for breeding stress-resistant tomato varieties.

RESULTS: Expression analysis revealed that low-temperature, drought, and abscisic acid (ABA) treatments significantly induced the expression of *SlWRKY45*. Overexpression of *SlWRKY45* enhanced the resistance of tomato plants to drought and low-temperature stresses. Under drought and low-temperature conditions, the photosynthetic indices, antioxidant enzyme activities, and proline (Pro) contents in *SlWRKY45* overexpression lines were significantly higher than those in wild-type (WT) plants. Conversely, the accumulation of reactive oxygen species (ROS) and malondialdehyde (MDA) levels in *SlWRKY45*-OE plants was significantly lower than in WT plants under the same stress conditions. Transcriptome data analysis indicated that *SlWRKY45* regulates tomato's response to low-temperature stress primarily by influencing antioxidant enzyme activities and stress response pathways. Dual-luciferase assays demonstrated that SlWRKY45 could directly activate the expression of SlPOD1. Furthermore, the interaction between SlWRKY45 and SlWRKY46 was confirmed through yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays.

CONCLUSION: Our findings demonstrate that SlWRKY45 positively regulates drought resistance and low-temperature tolerance in tomato. Additionally, SlWRKY45 can interact with SlWRKY46 and directly activate the expression of SlPOD1.

These results offer valuable insights for further research into the regulatory mechanisms underlying abiotic stress responses and provide potential gene resources for genetic improvement through molecular breeding.



Phenotypes of *SlWRKY45*-overexpressing and wild-type plants under drought and low-temperature treatments

Key words tomato, *SlWRKY45*, drought stress, low-temperature stress, antioxidase

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附表1 本研究所用引物
Appendix table 1 The primers used in this study

附图1 *SlWRKY45*的编码序列(CDS)克隆**(A)**、WRKY蛋白序列的多序列比对**(B)**和系统进化树**(C)**
蓝色线条区域代表WRKY结构域。红色星号代表高度保守的氨基酸位点。
Appendix figure 1 Coding sequence (CDS) cloning of *SlWRKY45* **(A)**, multiple alignment **(B)** and phylogenetic tree **(C)** of WRKY proteins
The blue line represents the WRKY domain. Red asterisks represent highly conserved amino acids.

附图2 *SlWRKY45*-OE转基因番茄株系的PCR **(A)**和表达**(B)**鉴定
(B) 以WT中*SlWRKY45*的相对表达量为对照。WT: 野生型。#1、#2、#5和#7为不同的转基因株系。**表示在*P*<0.01水平上差异显著。
Appendix figure 2 PCR **(A)** and expression **(B)** confirmation of *SlWRKY45* overexpressing lines
(B) The relative expression level of *SlWRKY45* in the WT was used as control. WT: Wild type. #1, #2, #5 and #7 are different transgenic lines. ** represents significant differences at *P*<0.01.

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