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·农业生物技术·

沉默大豆 *GmWRKY33B* 基因导致大豆抗病性降低

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摘　要：WRKY 转录因子基因家族是植物特有的转录因子，在防御中起着重要作用。通过生物信息学分析，本研究在古四倍体大豆(*Glycine max*)基因组中找到一对同源性高达 93%的 *WRKY33* 同源基因，并将其命名为 *GmWRKY33B*。从 *GmWRKY33B* 的两个同源基因保守区域选取一个 315 bp 片段构建至菜豆豆荚斑驳病毒(bean pod mosaic virus, BPMV)沉默载体(BPMV-VIGS)上，以期同时沉默上述 2 个 *GmWRKY33B* 基因。结果表明，同时沉默 2 个 *GmWRKY33B* 基因并不显著改变沉默植株的表型，但却显著降低了大豆对大豆斑点病菌以及大豆花叶病毒的抗性，说明 *GmWRKY33B* 在大豆免疫反应中起正调控作用。激酶分析表明，*GmWRKY33B* 沉默植株中 flg22 诱导的 *Gm*MPK6 的磷酸化水平较空载体 BPMV-0 植株显著降低，说明 *Gm*WRKY33B 可以通过调控 *Gm*MPK6 的激酶活性而参与大豆的免疫反应。抗毒素为大豆中主要起防御作用的植保素，而大豆异黄酮类特异性异戊烯基转移酶(prenyltransferase, PT)基因家族是参与大豆抗毒素生物合成的主要基因，许多 PT 基因启动子区含有与 WRKY 特异性结合的 W-box 序列。在丁香假单胞菌 pv.甘氨酸(*Pseudomonas syringae* pv. *glycinea*, *Psg*)侵染条件下，4 个 PT 基因的表达水平在沉默株系中显著降低，说明 *Gm*WRKY33B 参与 PT 基因的转录激活。综上所述，*Gm*WRKY33B 通过调控 *Gm*MPK6 的激活以及调控大豆抗毒素生物合成途径中关键酶编码基因的表达而参与免疫反应。

关键词：*GmWRKY33*；利用病毒诱导基因沉默；免疫反应；*Gm*MPK3/6；异戊烯基转移酶；大豆抗毒素

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Silencing *GmWRKY33B* genes leads to reduced disease resistance in soybean

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Abstract: The WRKYs are a group of plant-specific transcription factors that play important roles in defense responses. In this study, we silenced 2 *GmWRKY33B* homologous genes using a bean pod mosaic virus (BPMV) vector carrying a single fragment from the conserved region of the *GmWRKY33B* genes. Silencing *GmWRKY33B* did not result in morphological changes. However, significantly reduced resistances to *Pseudomonas syringae* pv. *glycinea* (*Psg*) and soybean mosaic virus (SMV) were observed in the *GmWRKY33B*-silenced plants, indicating a positive role of the *GmWRKY33B* genes in disease resistance. Kinase assay showed that silencing the *GmWRKY33B* genes significantly reduced the activation of *Gm*MPK6, but not *Gm*MPK3, in response to flg22 treatment. Reverse transcriptase PCR (RT-PCR) analysis of the genes encoding prenyltransferases (PTs), which are the key enzymes in the biosynthesis of glyceollin, showed that the *Psg*-induced expression of these genes was significantly reduced in the *GmWRKY33B*-silenced plants compared with the BPMV-0 empty vector plants, which correlated with the presence of the W-boxes in the promoter regions of these genes. Taken together, our results suggest that *Gm*WRKY33Bs are involved in soybean immunity through regulating the activation of the kinase activity of *Gm*MPK6 as well as through regulating the expression of the key genes encoding the biosynthesis of glyceollins.

Keywords: *GmWRKY33*; virus-induced gene silencing (VIGS); immune response; *Gm*MPK3/6; prenyltransferases; glyceollin

丝裂原活化蛋白激酶(mitogen-activated protein kinase, MAPK)信号转导途径在植物免疫反应的调控中起着重要作用[1-3]。植物细胞膜表面受体激酶或类受体蛋白在识别病原菌相关分子模式(pathogen-associated molecular patterns, PAMPs)或刺激信号后,激活 MAPK 级联反应[1-2]。MAPK 激酶信号转导途径可将无数外部刺激转化成细胞内的应答反应。WRKY 是植物特有的转录因子家族,可以特异性结合靶基因启动子区域的 W-box 顺式元件(TTGACC/T)[4-5]以调控基因表达,在植物防御反应中起着重要作用[6-9]。在拟南芥中,某些 WRKY 成员是 MPK3/MPK6 激酶的靶蛋白,WRKY 蛋白的磷酸化可以改变其结合 W-box 的能力从而影响其调控下游基因表达[10]。在真菌侵染条件下,MPK3/MPK6 对 WRKY33 的磷酸化是其激活植保素(camalexin)生物合成途径中编码关键酶基因所必需的[11-12]。与此类似,MPK3/MPK6 还可通过磷酸化 ERF6、MYB41 和 MYB44 等转录因子分别调控抗真菌、抗盐以及种子萌发等[2,13-14]。另外,拟南芥 MPK4 可以通过磷酸化 MKS1 (MAP kinase 4 substrate 1)[15]而解除其对 WRKY33 的抑制效应[16],

从而使 WRKY33 激活 *PAD3* 等基因的表达，继而增强植保素的生物合成[16-17]。WRKY 在大豆防御中也起着重要作用[18-20]。但这些 WRKY 是否受 MAPK 激酶途径调控以及是否参与大豆植保素的生物合成仍有待研究。

葡糖异硫氰酸盐(glucosinolates, GSs)包括吲哚葡糖异硫氰酸盐(indole glucosinolates, IGSs)，是十字花科植物中重要的次生代谢物，在免疫中起着重要作用[19,21]。MYB 转录因子参与调控 IGS 生物合成途径中基因的表达[21-22]。MPK3/MPK6 可调控 MYB51 与 MYB122 编码基因的表达；MPK3/MPK6 可通过其底物 ERF6 调控 CYP81F2 及 IGMT1/IGMT2 编码基因的表达，CYP81F2 及 IGMT1/IGMT2 是将吲哚-3-基甲基硫代葡萄糖苷(indole-3-yl-methylglucosinolate, I3G)转化成具抑菌效应的 4-甲氧基吲哚-3-基甲基硫代葡萄糖苷(4-methoxyindole-3-yl-methylglucosinolate, 4MI3G)的关键酶[23]。此项研究表明 MPK3/MPK6 可通过调控下游 2 个转录因子协同完成植保素 IGS 的生物合成。

与拟南芥不同，大豆中的主要植保素是大豆抗毒素(glyceollin)。大豆抗毒素是一组拥有紫檀碱(pterocarpan)骨架及不同 C_5 基团经异戊烯基化修饰的次生代谢物[24]，经不同异戊烯基转移酶(prenyltransferase, PT)催化而成[24]。源于同一前体物的大豆抗毒素衍生物可分为 I–V 类，但 I–III 类是主要的植保素[25]。不同病原菌侵染可诱导大豆合成大豆抗毒素[26]。通过转基因策略提高病原菌诱导的大豆抗毒素合成可增强大豆的抗病性[27-31]。但大豆抗毒素生物合成的调控机理仍不清楚。MAPK 途径是否参与其合成的调控、哪个(些)转录因子参与其生物合成途径中关键基因的表达调控也未见报道。

通过菜豆斑驳病毒(bean pod mottle virus, BPMV)介导的病毒诱导基因沉默(virus-induced gene silencing, VIGS)技术同时沉默两个 *GmWRKY33B*，导致大豆对大豆花叶病毒及大豆斑点病菌[丁香假单胞菌 pv.甘氨酸(*Pseudomonas syringae* pv. *glycinea*, *Psg*)]抗性降低、flg22 诱导的 *Gm*MPK6 激活程度降低以及 *Psg* 诱导的负责大豆抗毒素生物合成的异戊烯基转移酶 PT 基因表达水平的显著降低，说明 *Gm*WRKY33B 为大豆免疫反应的正调控因子。

1 材料与方法

1.1 材料

1.1.1 大豆

本研究中使用的大豆(*Glycine max*)为 Williams 82。大豆植株在温室或生长室中保持在 22 ℃下，光周期为 16 h。

1.1.2 菌种

大肠杆菌(*Escherichia coli*) DH5α/TOP10、*Pseudomonas syringae* pv. *glycinea* (*Psg*) R4 菌株以及 SMV-N-GUS[32]。

1.1.3 flg22

源自 *Psg* 的 flg22 小肽由杭州华安生物技术有限公司合成[33-34]。

1.2 方法

1.2.1 BPMV 介导的 VIGS 技术-载体构建、基因枪法侵染及沉默效果验证

对于 BPMV-VIGS 载体系统以及如何使用 PDS-1000/He (Bio-Rad 实验室)基因枪法将 BPMV 接种到大豆幼苗上在此前已有描述[35-36]。通过在 Phytozome 基因组数据库(https://phytozome-next.jgi.doe.gov/Phytozome)中进行关键词搜索，确定了 2 个大豆 *GmWRKY33B* 同源基因：Glyma.09G280200 以及 Glyma.18G208800。首先用 Glyma.09G280200-F 与 Glyma.09G280200-R 这对引物(表 1)从大豆 cDNA 中扩增出 315 bp 的片段，接着用 *Bam*H I 与 *Kpn* I 对该扩增片段进行双酶切，然后连接至经同样双酶切的 BPMV2 载体中，构建成可

表 1 本研究所用的引物

Table 1 Primers used in this study

Primer name	Primer sequence (5′→3′)	Size (bp)
Construction of primers for BPMV-VIGS vector		
Glyma.09G280200-F	AAA**GGATCC**ATAAAATATGGACAGAAACAAGTGA	34
Glyma.09G280200-R	AAA**GGTACC**CTTCCTCTCCAACAGAAGCTG	30
Primers for silencing examination		
Glyma.09G280200-F	ACCTTTCAACCAATAATAAACAA	23
Glyma.09G280200-R	ATTAGAAAGAGCATAGTAGAAATAG	25
Glyma.18G208800-F	ACCTTCTTTCCATCAATAATAAAG	24
Glyma.18G208800-R	TAAAAGTAGAAATTGCCAAAAC	22
Primers for RT-PCR		
Glyma.10G295300.1-F	TGTGCTGCCTCAATTGTTTA **(G4DT)**	20
Glyma.10G295300.1-R	TGCAAGAGCCCAAGTAGAA	19
Glyma.01G134600.1-R	AATGTAATTGGCAATACAAATG	22
Glyma.13G097800-F	TAACAACGCTTCTTCTCTCG **(HPT)**	20
Glyma.13G97800-R	AAGGAATTCTTGACAGAGTCC	21
Glyma.10G070200-F	CATAATCATTGCTGTGAGTACTG **(C4DT)**	23
Glyma.10G070200-R	CAAGAACCTATAACCAAGCTAAG	23
GmELF1b-F	GAGCTATGAATTGCCTGATGG	21
G*mELF1b*-R	CGTTTCATGAATTCCAGTAGC	21
Primers for RT-qPCR		
Glyma.10G295300-F	TCTACTCCTTGGGTTTGG	18
Glyma.10G295300-R	CCCGTTTCTGACCTAGAC	18
Glyma.10G070200-F	AAGCAGCCCATGCTATAC	18
Glyma.10G070200-R	CCAGTACAAAGCAACCAAG	19
Glyma.01G134600-F	ACCTCATACTTCCAATCCTC	20
Glyma.01G134600-R	GCAAGGAGAGACGAAGAA	18
Glyma.13G097800-F	GCTTCATGGCACAATAGG	18
Glyma.13G097800-R	CATGTACACCCTCCTTCA	18
GmELF1b-F	GTTGAAAAGCCAGGGGACA	19
GmELF1b-R	TCTTACCCCTTGAGCGTGG	19

The bold sequences are *Bam*H I and *Kpn* I restriction sites, for cloning PCR fragments into BPMV-VIGS.

同时沉默 Glyma.09G280200 和 Glyma.18G208800 的 BPMV 沉默载体 BPMV2-*GmWRKY33B*。

载体构建成功后，将 BPMV2 空载体和 BPMV2-*GmWRKY33B* 质粒分别与 BPMV1 质粒混合包被在 1.6 μm 金粉颗粒上，利用 PDS-1000/He 基因枪将上述金粉颗粒分别轰至生长 7 d 左右大豆幼苗两片展开的真叶上；3 周左右，侵染成功的大豆植株的上部系统叶片便会出现病毒症状。在有症状的系统叶片打孔取样后提取 RNA 进行沉默效果的验证。验证成功后，将所有具病毒症状的系统叶片收集后在磷酸缓冲液中研磨，离心后含大量病毒的汁液–80 ℃储存，用于后续大规模摩擦接种。

1.2.2 RT-PCR

cDNA 的合成：总 RNA 的提取按生产商提供的方法进行(Invitrogen)；cDNA 的合成按厂商提供的方法(ReverTra Ace qPCR RT Kit, TOYOBO)进行；逆转录反应为：将 RNA 在 65 ℃热变性 5 min，置于冰上。然后在离心管里顺次加入：2 μL 5×RT Buffer；0.5 μL Primer Mix；0.5 μL RT

Enzyme Mix；1.0 μg RNA；最后用不含核酸酶的水将终体积调至 10.0 μL；逆转录反应在 37 ℃下进行 15 min；98 ℃变性 5 min；将 cDNA –20 ℃储存备用。

PCR 反应：PCR 反应按 Tian 等[34]所述方法进行。PCR 扩增程序为：95 ℃预变性 2 min；95 ℃ 15 s，56 ℃ 30 s，72 ℃ 20 s，35 个循环；72 ℃延伸 5 min。用 SYBR green qPCR RT 试剂盒(TOYOBO)进行实时定量聚合酶链式反应(quantitative real-time polymerase chain reaction, qPCR)，qPCR 反应按生产商提供的操作手册进行。qPCR 扩增所用仪器为 Applied Biosystems Quantstudio 1 (Thermofisher)。qPCR 扩增程序为：95 ℃预变性 2 min；95 ℃ 15 s，52 ℃ 20 s，72 ℃ 30 s，共 40 个循环；72 ℃延伸 5 min。

1.2.3 沉默效果验证

分别用 Glyma.09G280200 与 Glyma.18G208800 的沉默验证引物(表 1)对从沉默植株中提取的 RNA 所合成的 cDNA 进行 RT-PCR 分析。所用正向与反向沉默引物分别位于构建沉默载体所用插入片段上、下游 200 bp 左右的位置。

1.2.4 接种大豆斑点病病菌及菌落单位计数分析

Psg 的培养、接菌及菌落计数分析按文献中所描述方法进行[34,37]。

1.2.5 SMV-N-GUS 接种、GUS 染色和 GUS 病灶测量

SMV-N-GUS 的侵染、GUS (β-glucuronidase)染色和 GUS 病灶测量已有描述[32]。通过基因枪法将与 GUS 报告基因融合的 SMV-N 菌株(SMV-N-GUS)分别接种到空载体对照植株(BPMV-0)与 BPMV-*GmWRKY33B* 沉默植株离体叶片中[33,38-40]；接着将被侵染的离体叶片置于铺有湿润滤纸的培养皿中于室温下培养 3 d 后进行 GUS 染色。使用显微镜(Olympus)对 GUS 染色情况进行观察并拍照；并用 Image J 软件测量 GUS 病斑直径。

1.2.6 免疫印迹法分析检测磷酸化 *Gm*MPK3/6

从大豆叶片组织中提取蛋白质按以前发表的方法进行[33-34,38,40]。蛋白提取液组分为：50 mmol/L Tris-MES (pH 8.0)，0.5 mol/L sucrose，1 mmol/L $MgCl_2$，10 mmol/L EDTA，5 mmol/L DTT，另加蛋白酶抑制剂混合液(protease inhibitor cocktail, Sigma-Aldrich)。将从 flg22 处理后的叶片中提取的蛋白通过 SDS-PAGE 胶进行分离，然后用半干电转移装置(Bio-Rad)将胶中的蛋白转移至 PVDF 膜上(Millipore)；将转移后的 PVDF 膜在 TBST 缓冲液中封闭，然后用 1:3 000 稀释的 p44/p42 抗体(cell signaling technology)进行孵育；一抗孵育后的膜用 TBST 清洗 3 次，然后用 1:7 500 稀释的二抗进行孵育。最后，用化学发光的辣根过氧化物酶(horseradish peroxidase, HRP)底物，即过氧化物溶液和增强型鲁米诺溶液的混合液(Millipore)通过曝光检测条带。

2 结果与分析

2.1 大豆 *GmWRKY33* 同源基因基之间同源性关系分析

利用拟南芥 *WRKY33* 基因编码区序列对大豆基因组进行 BLAST (phytozome 13)，结果表明大豆基因组中有 6 个 *WRKY33* 同源基因：Glyma.02G232600、Glyma.11G163300、Glyma.14G200200、Glyma.18G056600、Glyma.09G280200 以及 Glyma.18G208800。对这 6 个 *GmWRKY33* 同源基因编码序列的进化分析表明，这 6 个基因可分为 3 组(图 1)，组内 2 个基因之间同源性高达 91%以上。由于前 4 个基因之间的同源性高达 84%，因此将前 4 个基因统称为 *GmWRKY33A*，而将后 2 个同源性高达 95%但却与 *GmWRKY33A* 不同成员间同源性仅为 58%–68%的基因统称为 *GmWRKY33B*[41] (表 2)。

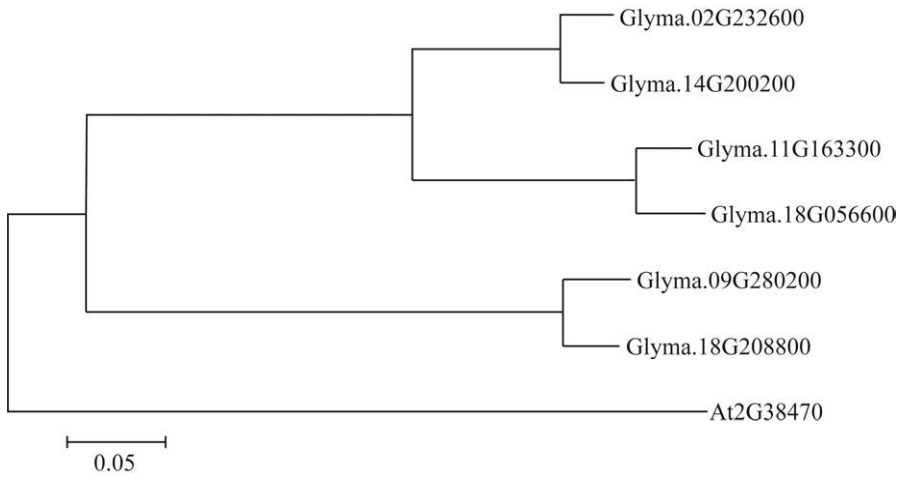

图 1　大豆 6 个 *GmWRKY33* 基因编码区序列的进化聚类分析

Figure 1　Phylogenetic analysis of the sequences of the coding regions of six *GmWRKY33* genes discovered in soybean. The *Arabidopsis WRKY33* gene (At2G38470) was used as a reference gene. The software used for phylogenetic analysis was MEGA 5.10.

表 2　大豆 *GmWRKY33* 同源基因的同源性比较

Table 2　Comparison of homology between *GmWRKY33* homologs in soybean

Gene ID	Glyma. 02G232600	Glyma. 14G200200	Glyma. 11G163300	Glyma. 18G056600	Glyma. 09G280200	Glyma. 18G208800
Glyma.02G232600 silencing fragment (%)	100	97	85	85	69	70
Glyma.09G280200 silencing fragment (%)	67	68	58	59	100	95

The first four genes are *GmWRKY33A*, and the last two genes are *GmWRKY33B*.

为了探讨 *Gm*WRKY33B 的功能，构建了携带 Glyma.09G280200 与 Glyma.18G208800 保守区 315 bp 片段的 BPMV-VIGS 载体，以期同时沉默 2 个 *GmWRKY33B* 基因。用上述构建好的 *GmWRKY33B* 沉默载体侵染大豆幼苗。侵染后 15 d 左右便会观察到病毒症状。接种 BPMV-0 空载体的对照植株与 BPMV-*GmWRKY33B* 沉默植株在表型上无显著差异(图 2A)，说明同时沉默这 2 个基因对大豆的生长发育并无影响。RT-PCR 验证分析表明，*GmWRKY33B* 沉默植株中 Glyma.09G280200 与 Glyma.18G208800 这 2 个基因的转录水平较 BPMV-0 空载体对照植株均显著降低(图 2B)，说明这 2 个基因被同时沉默，进一步证实了 BPMV-VIGS 在克服基因功能冗余的优越性[42-43]。

2.2　同时沉默 2 个 *GmWRKY33B* 基因显著降低对大豆斑点病菌(*Psg*)的抗性

为了检测同时沉默 2 个 *GmWRKY33B* 基因对 *Psg* 抗性的效应，用 *Psg* R4 菌株对空载体对照植株及 *GmWRKY33B* 沉默植株系进行菌落计数分析。结果表明，沉默植株叶片上的病症均较 BPMV-0 叶片上严重(图 3A)；与发病症状结果相一致，接菌不同时间后 *GmWRKY33B* 沉默植株叶片中 *Psg* 繁殖数显著高于 BPMV-0 叶片(图 3B)，说明同时沉默 *GmWRKY33B* 增加了大豆对 *Psg* 侵染的敏感性。

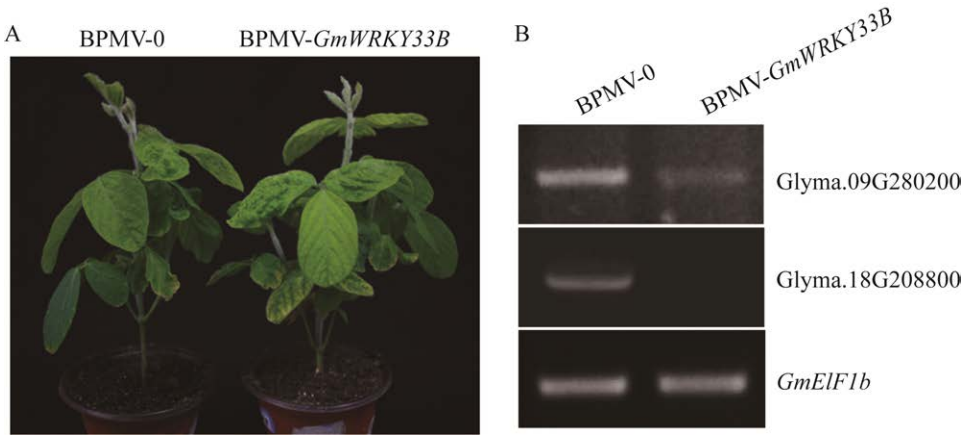

图 2　同时沉默 2 个 *GmWRKY33B* 基因未导致大豆表型变化

Figure 2　Silencing 2 *GmWRKY33B* genes simultaneously does not result in morphological changes in soybean. A: Comparisons of the morphological phenotypes between the *GmWRKY33B*-silenced plants and the empty vector plants. B: The transcript levels of the two *GmWRKY33B* genes were significantly reduced in *GmWRKY33B*-silenced plants relative to empty vector control plants.

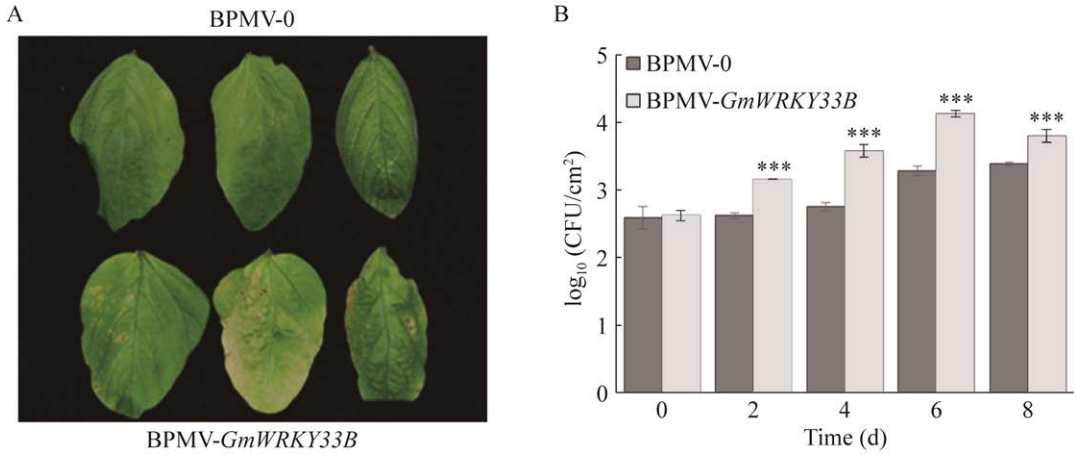

图 3　BPMV-0 和 *GmWRKY33B* 沉默株系接种 *Psg* 后的表型及菌落形成单位比较

Figure 3　Comparison of the symptom severity and the growth rates between the BPMV-0 plants and the *GmWRKY33B*-silenced plants infected by *Psg* R4 strain. A: The bacterial symptoms on the leaves of the *GmWRKY33B*-silenced and BPMV-0 plants 8 days post *Psg* inoculation. B: The bacterial growth assays on the BPMV-0 and the *GmWRKY33B*-silenced plants after *Psg* inoculation for the indicated time. CFU represents colony forming unit. The unit for *Y* axis means the $\log_{10}$ number of colonies replicated in 1 cm^2 leaf area. Standard deviation, ***: $P<0.001$ (Student's *t*-test).

2.3　同时沉默 2 个 *GmWRKY33B* 基因显著降低大豆对 SMV 的抗性

钟晨丽[41]证明同时沉默 4 个 *GmWRKY33A* 基因可降低大豆中对 SMV 的抗性。为了检测同时沉默 2 个 *GmWRKY33B* 基因对 SMV 抗性的效应，将 SMV-N-GUS (与报告基因 GUS 融合的 SMV N 毒株)分别接种至 BPMV-0 与 *GmWRKY33B* 沉默植株的离体叶片上，在室温下培养 3 d 后进

行 GUS 染色。染色后蓝色斑点的出现意味着侵染成功，而 GUS 斑点的大小代表病毒复制和/或运动的程度。结果表明 *GmWRKY33B* 沉默植株系叶片中的 GUS 斑点的直径的大小及染色强度均显著高于空载体植株(图 4)，说明同时沉默 2 个 *GmWRKY33B* 同源基因可显著降低大豆对 SMV-N-GUS 的抗性。

2.4 沉默 *GmWRKY33B* 降低了 flg22 诱导的 *Gm*MPK3/6 的激活程度

Flg22 为细菌鞭毛蛋白 N 端的含 22 个氨基酸的小肽，其作为 PAMP 可被定位于质膜上的受体激酶或受体蛋白识别而激活免疫反应[44]。激活的受体可通过磷酸化激活 MAPK 信号转导途径，而激活的 MPK3/6 可通过磷酸化 WRKY 等转录因子以激活防御相关基因[2,12,45]。前期研究表明源自 *Psg* 与 *Pst* (*Pseudomonas syringae* pv. tomato, DC3000)的 flg22 均可诱导大豆 *Gm*MPK3/6 的激活[34]。为了检测大豆中 flg22 诱导的 *Gm*MPK3/6 的激活是否依赖于 *Gm*WRKY33B，用 10 μmol/L 源自 *Psg* 的 flg22 分别处理 BPMV-0

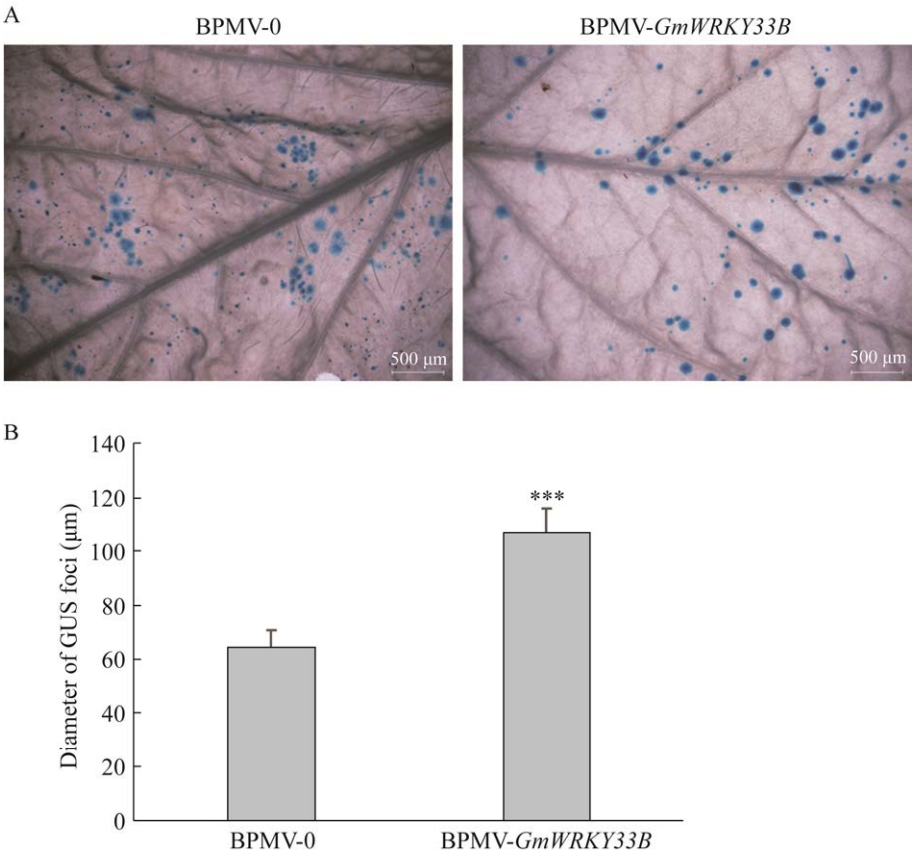

图 4 同时沉默 2 个 *GmWRKY33B* 基因导致大豆对 SMV-N-GUS 抗性的降低

Figure 4 Silencing 2 *GmWRKY33B* genes simultaneously leads to the reduced resistance of soybean to SMV-N-GUS. A: Comparisons of the GUS foci on the leaves of the BPMV-0 and the BPMV-*GmWRKY33B* plants under a microscopy. Bar=5 mm. B: Comparisons of the diameters of the SMV-N-GUS foci on the leaves of BPMV-0 and BPMV-*GmWRKY33B* plants. The error bars indicate that the standard deviation calculated by measuring at least 30 lesions (≥120 lesions) in 4 independent leaves; ***: $P<0.001$ (Student's *t*-test).

空载体与 *GmWRKY33B* 沉默株叶片，在侵染不同时间取样并提取蛋白，并用专一性识别磷酸化 MPK3/4/6 的抗体 Phospho-p44/42 MAP Erk1/2 进行激酶分析。结果表明沉默 *GmWRKY33B* 显著降低了 flg22 对 *Gm*MPK6 的激活程度(图 5)。虽然 *Gm*MPK3 在 BPMV-0 空载体植株与 *GmWRKY33B* 沉默植株中激活水平均很低，但在 *GmWRKY33B* 沉默植株中激活程度略低于 BPMV-0 空载体植株(图 5)，表明 *Gm*MPK6 而非 *Gm*MPK3 的诱导激活依赖于 *Gm*WRKY33B。

2.5 沉默 *GmWRKY33B* 导致 *Psg* 诱导的大豆抗毒素生物合成相关基因表达水平降低

大豆中主要植保素为大豆抗毒素[25]，而拟南芥中的主要植保素为亚麻荠素(camalexin)[11,23]，它们的生物合成途径不同。大豆异黄酮类特异性异戊烯基转移酶基因家族在大豆抗毒素的生物合成中起着关键作用[25]。对与大豆抗毒素生物合成密切相关的 11 个异黄酮类特异性异戊烯基转移酶基因[25]启动子区 1.5 kb 左右的序列分析，发现这些基因的启动子区含有 1–5 个 W-box 序列(TTGACC/T)，暗示这些基因的表达可能受 *Gm*WRKY 调控。通过 RT-PCR 和 RT-qPCR 分析比较 BPMV-0 空载体对照植株与 *GmWRKY33B* 沉默植株叶片受 *Pst* 侵染 48 h 后 *GmHPT*、*GmG2DT*、*GmG4DT* 和 *GmIDT3* 的诱导表达情况。结果表明，沉默 *GmWRKY33B* 导致上述基因诱导表达水平显著降低(图 6A、6B)，说明 *GmWRKY33B* 基因家族参与调控这些基因的诱导表达，进而参与大豆抗毒素的生物合成。

3 讨论与结论

WRKY 是高等植物特有的转录因子，在植物免疫反应中起着重要作用[12,46-47]。大豆是古四倍体植物，75%以上的基因存在功能冗余的情况[48]，用传统筛选突变体的方法不适用于大豆基因功能的研究。而 VIGS 技术恰好可弥补这一缺陷。VIGS 技术可以同时沉默同源性高达 85%以上的基因，可以克服大豆基因功能冗余的障碍[42-43]。通过 BPMV-VIGS 技术利用单个沉默载体同时沉默了 2 个 *GmWRKY33B* 基因

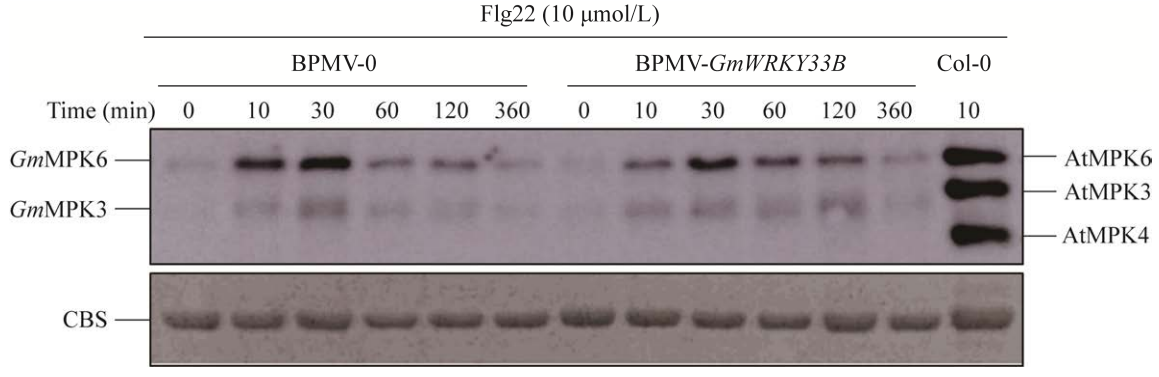

图 5 沉默 *GmWRKY33B* 同源基因降低了大豆应对 flg22 诱导的 *Gm*MPK3/6 激酶的激活程度

Figure 5 The activation of *Gm*MPK3 and *Gm*MPK6 in response to flg22 is significantly reduced in *GmWRKY33B*-silenced plants. Leaf discs from the BPMV-0 and *GmWRKY33B*-silenced plants were incubated on a moist filter paper for 24 h to allow recovery from wounding before being treated with 10 μmol/L flg22 derived from *Psg* over the indicated time points. The kinase activities were detected by Western blotting using Phosphp44/p42 MAP Erk1/2 antibody, which specifically recognizes phosphorylated MPK3/4/6 across the kingdoms. Coomassie blue stained gel (CBS) was used as loading controls. The *Arabidopsis* sample treated with flg22 for 10 min was used as a positive control.

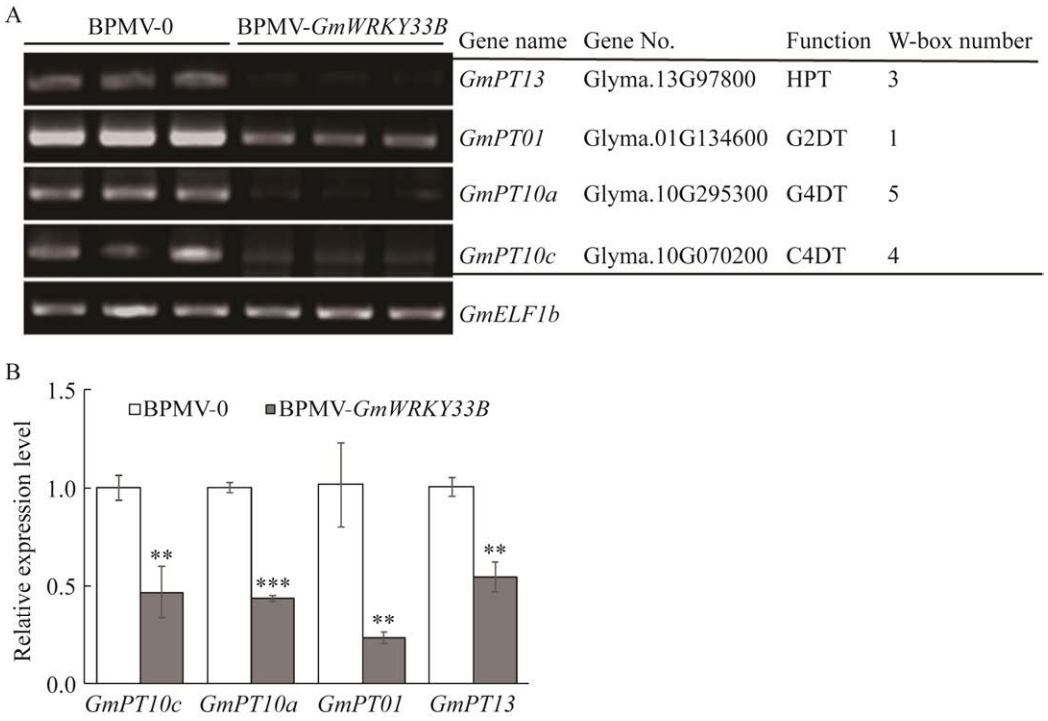

图 6　细菌侵染对 *GmPT* 基因表达效应的分析

Figure 6　RT-PCR and RT-qPCR analysis of the *GmPT* genes in response to *Psg* infection. The BPMV-0 and *GmWRKY33B*-silenced plants were infected with *Psg* for 48 hours, and the RNA was subsequently isolated from the infected leaves. RT-PCR (A) and RT-qPCR (B) were performed to monitor the expression of prenyltransferases genes. *GmElF1b* was used as an internal reference gene. The name, Glyma number, protein function and the number of W-box presented in the promoter region for each gene are listed on the right. The enzyme names and abbreviations encoded by the *PT* genes are: G2DT: Glycinol 2-dimethylallyltransferase; C4DT: Glycinol 4-dimethylallyltransferase coumestrol 4-dimethylallyltransferase; HPT: Homogentisate phytyltransferase. ** and *** represent $P<0.01$ and $P<0.001$, respectively, by Student's *t*-tests.

(图 2)。抗病性鉴定结果表明沉默 *GmWRKY33B* 可降低大豆对 *Psg* 及 SMV-N-GUS 的抗性(图 3，图 4)，说明 *Gm*WRKY33B 基因家族在大豆免疫反应中起着重要作用。通过在保守区构建携带单一 Glyma.02G232600 片段的 BPMV-VIGS 载体成功同时沉默 *GmWRKY33A* 的 4 个同源基因，并证明其在大豆防御中起着正调控作用[41]。拟南芥 WRKY33 通过调控植保素生物合成关键基因的表达而参与大豆植保素的生物合成[12]；在油菜中过表达 BnWRKY33 可增强对 *Sclerotinia sclerotiorum* 的抗性[49]，说明不同植物的 WRKY33 在抗性方面的功能是保守的。

当拟南芥受到病原体侵害时，其表面模式识别受体可识别 PAMPs 并被激活，激活的受体复合体可将磷酸化信号传递给 MKK4/5-MPK3/6，然后通过 MPK3/6 磷酸化 WRKY33，磷酸化后的 WRKY33 能够启动下游植保素合成相关基因的表达[12]。激酶分析结果表明在大豆中沉默 *GmWRKY33B* 显著降低了 flg22 诱导的 *Gm*MPK6 的激活程度(图 5)，说明 *Gm*WRKY33B 的功能是 *Gm*MPK3/6 激活所必需的，亦即 *Gm*WRKY33B 是大豆 PAMP 触发的免疫力(PAMP-triggered

immunity, PTI)途径的必要组分。有研究表明在胁迫条件下，拟南芥 MPK3/MPK6 的激活可诱导 ACC 合成酶与 WRKY 的表达，而诱导表达的 WRKY 又可上调 MPK3/MPK6 的表达，从而形成一个正反馈环调控 ACC 合成酶的表达及乙烯的合成[50]。另外，MPK3/MPK6 通过磷酸化 ERF 转录因子调控防御基因的表达及对真菌的抗性[45]。与此相类似，推测 *Gm*MPK6 可以激活 *Gm*WRKY33B 的转录活性；反过来，*Gm*WRKY33B 又可调控 *GmMPK6* 基因的表达，二者之间形成一个正反馈调控环路促进防御相关基因的表达。

异黄酮是大豆中广泛存在的黄酮类次生代谢物，而异黄酮通过异戊烯基转移酶羟基化以及进一步环化形成含有一个或多个 C5 或 C10 芳香环的大豆抗毒素[51-52]，目前有 11 个特异性的异戊烯基转移酶编码基因已被证实参与大豆抗毒素的生物合成[25]。发现这 11 个基因启动子区含有 1–5 个 W-box，暗示这些基因可能受 WRKY 调控。比较了在 *Psg* 侵染条件下 PT 基因在 *GmWRKY33B* 沉默植株与 BPMV-0 空载体植株间诱导表达的差异，结果发现沉默这些 *GmWRKY33B* 显著降低了大豆抗毒素生物合成途径中这 4 个关键酶编码基因的表达(图 6)，说明 *Gm*WRKY33B 可通过调控大豆抗毒素的生物合成而参与防御反应。接下来通过高效液相色谱测定在病原菌侵染条件下 *GmWRKY33B* 沉默植株与 BPMV-0 空载体植株中的抗毒素含量便可验证推测。如果测定结果如所期，将创制过表达 *GmWRKY33B* 的转基因株系，为抗性育种提供材料。

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