The *Meloidogyne javanica* effector Mj2G02 interferes with jasmonic acid signalling to suppress cell death and promote parasitism in *Arabidopsis*

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**Abstract**

Plant-parasitic nematodes can cause devastating damage to crops. These nematodes secrete effectors that suppress the host immune responses to enhance their survival. In this study, Mj2G02, an effector from *Meloidogyne javanica*, is described. In situ hybridization and transcriptional analysis showed that Mj2G02 was highly expressed in the early infection stages and exclusively expressed in the nematode subventral oesophageal gland cells. In planta RNA interference targeting Mj2G02 impaired *M. javanica* parasitism, and Mj2G02-transgenic *Arabidopsis* lines displayed more susceptibility to *M. javanica*. Using an *Agrobacterium*-mediated transient expression system and plant immune response assays, we demonstrated that Mj2G02 localized in the plant cell nuclei and could suppress Gpa2/RBP-1-induced cell death. Moreover, by RNA-Seq and quantitative reverse transcription PCR analyses, we showed that Mj2G02 was capable of interfering with the host jasmonic acid (JA) signalling pathway. Multiple jasmonate ZIM-domain (JAZ) genes were significantly upregulated, whereas the JAR1 gene and four JA-responsive genes, MYC3, UPI, THI2.1, and WRKY75, were significantly downregulated. In addition, HPLC analysis showed that the endogenous jasmonoyl-isoleucine (JA-Ile) level in Mj2G02-transgenic *Arabidopsis* lines was significantly decreased compared to that in wildtype plants. Our results indicate that the *M. javanica* effector Mj2G02 suppresses the plant immune response, therefore facilitating nematode parasitism. This process is probably mediated by a JA-Ile reduction and JAZ enhancement to repress JA-responsive genes.

**KEYWORDS**
cell death suppression, effector, JA-Ile, JA-responsive genes JAZ genes, *Meloidogyne javanica*

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Plant-parasitic nematodes (PPNs) are economically important pests as they cause more than $80 billion in losses in world agricultural yield every year (Mantelin et al., 2017). Root-knot nematodes (RKNs), *Meloidogyne* spp., are among the most economically devastating PPNs, infecting more than 5,500 plant species in all continents (Bloks et al., 2008). As a sedentary parasite, infective second-stage juveniles of RKNs penetrate the host root elongation zone and enter the vascular tissues, where they induce the formation of multinucleated giant cells. These giant cells constitute a source of nutrients for the RKNs (Davis et al., 2004). RKNs rely on sustained biotrophic interactions with host plants to establish and maintain the feeding sites. To do so, effector proteins synthesized in the nematode oesophageal glands usually play a role in the interaction, commonly suppressing plant defence responses and manipulating plant signalling pathways (Gheyseyn & Mitchum, 2011; Huang et al., 2004; Jaouannet & Rosso, 2013).

Over the past two decades, an increasing number of effectors have been isolated from RKNs using various strategies. In earlier years, 37 candidate pioneer effectors expressed in the oesophageal gland cells and 486 proteins from the secretome of *M. incognita* were obtained by two independent studies performing cDNA library and mass spectrometry screenings, respectively (Bellaiole et al., 2008; Huang et al., 2003). In recent years and thanks to technological advances, studies have identified hundreds of novel potential RKN effectors based on transcriptomes and genomes (Danchin et al., 2013; Nguyen et al., 2018; Petitot et al., 2016; Rutter et al., 2014). However, the roles of most of these effectors are still largely unknown. Therefore, it is imperative to explore their functions to better understand the molecular mechanism of nematode parasitism to develop new pest control methods.

Nucleus-targeted effectors often play critical roles during the plant-pathogen interaction as more and more evidence suggests that the cell nucleus is an essential target of numerous pathogen effectors (Mukhtar et al., 2011; Rivas, 2012; Rivas & Genin, 2011). It has been experimentally demonstrated by transient expression that several effectors with predicted nuclear localization signals (NLSs), from both RKNs and cyst nematodes (CNs), are localized in plant nuclei (Hewezi et al., 2015; Lin et al., 2013; Quentin et al., 2013; Zhang et al., 2015). Notably, some of these effectors were shown to interfere with host cellular processes, such as gene expression, plant immunity, and plant hormone signal transduction (Hewezi et al., 2015; Mejias et al., 2020; Pogorelko et al., 2019; Verma et al., 2018). Several notable examples are the *M. graminicola* effector MgGPP, the *M. incognita* effector MIEFF18, and the *Heterodera schachtii* effector Hs3OD08. Despite their similar localization, these effectors exert their action through different molecular mechanisms. MgGPP recruits host-mediated glycosylation and proteolysis, and is then translocated to the nuclei to suppress plant defence responses (Chen et al., 2017). MIEFF18 is secreted into the nuclei of giant cells and targeted to the plant core spliceosomal protein SmD1, promoting the development of giant cells (Mejias et al., 2020), and Hs3OD08 targets the plant nuclei and interacts with a host auxiliary spliceosomal protein SMU2, which leads to the alteration of the pre-mRNA splicing and gene expression in feeding sites (Verma et al., 2018).

In 2003, a study by Huang and colleagues obtained a profile of 37 cDNA sequences, including 2G02 encoding a putative parasitism protein expressed in the oesophageal gland cells of *M. incognita* (Huang et al., 2003). In this study, we aim to characterize a novel nuclear effector, Mj2G02 (an ortholog of *M. incognita* 2G02), from *M. javanica*. Herein, we provide evidence that the effector Mj2G02 enhances *M. javanica* parasitism and can interfere with the jasmonic acid (JA) signalling pathway to suppress plant immunity.

## Results

### 2.1 Sequence analysis of the Mj2G02 gene from *M. javanica*

First, we performed a sequence analysis of the full-length cDNA sequence of Mj2G02 (GenBank accession MW767382). Mj2G02 contained an open reading frame of 633 bp, encoding a 210 amino acid protein with a predicted 18 amino acid N-terminal signal peptide (Figure S1). According to InterProScan, a putative N-terminal ShK toxin (ShKt) domain (33–68 amino acids) and a coiled-coil region (123–143 amino acids) were present (Figure 1a). In addition, Mj2G02 contained two putative SV40-like NLS motifs, $^{62}$PKKKV$^{68}$ and $^{101}$KRRK$^{107}$ (Figure 1b). According to PSORT II, Mj2G02 was predicted to localize to the nucleus (Table S1).

When we performed a BLAST search against WormBase, we found that Mj2G02 shared a high degree of homology with proteins from various RKN species, including *M. incognita* (Minc3s00855g18130, 99.5%), *M. arenaria* (Scaf918g016851, 92.4%), *M. floridensis* (maker-nMf.1.1.scaf23378-augustus-gene-0.3, 89.0%), *M. enterolobii* (scaffold13260_cov183.g16249, 85.4%), *M. hapla* (MhA1_Contig2148.frz3, gene8, 75.8%), and *M. graminicola* (NXFT01000603.1.1405_g., 55.6%), but a low degree of homology with proteins from CNs, including *Heteroder a glycines* (Hetgly.G000014452, 46.2%) and *Globodera rostochiensis* (GROS_g02633.t1, 38.6%). A multiple sequence alignment of the deduced Mj2G02 amino acid sequences with PPN homologs is presented in Figure 1b. When searching against other released PPN genomes, as well as bioinformatics resources of other animal-parasitic and free-living nematodes, no significant matches were found.

### 2.2 Mj2G02 is expressed in the subventral oesophageal glands and upregulated in the invasion and early parasitic stages of *M. javanica*

Tissue localization of Mj2G02 was investigated by in situ hybridization using digoxigenin (DIG)-labelled Mj2G02 probes in pre-parasitic second-stage juveniles (pre-J2) of *M. javanica*. The result showed that Mj2G02 transcripts were specifically present in the...
subventral oesophageal gland cells after hybridization with the antisense probes. As expected, no signals were detected with the sense probes (Figure 2a).

Next, the expression of Mj2G02 was analysed at different developmental stages using quantitative reverse transcription PCR (RT-qPCR). The expression level of Mj2G02 at the egg stage was set to 1 to calculate the relative fold changes in other stages. As a result, the maximum expression level of Mj2G02 was detected in the pre-J2 stage, at approximately 26-fold compared with that in eggs. After the pre-J2 stage, Mj2G02 transcripts decreased gradually but remained at a high level (15-fold) at 2 days postinoculation (dpi) (Figure 2b).

These findings suggest that Mj2G02 may play a role in the invasion and migration of *M. javanica*.

### 2.3 Mj2G02 is localized in the plant cell nucleus

Enhanced green fluorescent protein (eGFP) and Mj2G02Δsp:eGFP were transiently expressed in Arabidopsis protoplast cells. After 48 hr, the GFP fluorescent signal of Mj2G02Δsp:eGFP was observed in the nuclei of transformed cells. In the control cells transformed with eGFP alone, the fluorescence signal was found in the cytoplasm.
and nuclei (Figure 3a). Western blotting using an anti-GFP antibody detected bands at approximately 49 and 27 kDa for Mj2G02Δsp:eGFP and eGFP alone, respectively (Figure 3b), indicating that the Mj2G02Δsp-eGFP fusion protein was intact. To further confirm these results in a different plant species, these fusion proteins were also transiently expressed in Nicotiana benthamiana leaves. Similarly, the GFP signal of Mj2G02Δsp:eGFP was observed in the cell nuclei, and the free eGFP was detected in both cytoplasm and nuclei of cells (Figure 3c).

2.4 | In planta RNA interference of Mj2G02 impairs M. javanica parasitism

To investigate whether Mj2G02 can affect M. javanica parasitism, host-mediated gene silencing was performed to silence Mj2G02 in the feeding nematodes. A β-glucuronidase (GUS) intron fragment of the RNA interference (RNAi) cassette was detected in four transgenic lines by RT-PCR (Figure S2a), suggesting that these transgenic lines may express Mj2G02 dsRNA. No GUS fragments were detected in wildtype (WT) plants and plants expressing empty vector (EV) as controls. The phenotype of transgenic lines had no apparent differences compared to control plants. Next, the transcription of Mj2G02 in nematodes was assessed by RT-qPCR. We found that the transcription of Mj2G02 from RNAi lines at 3 dpi was significantly lower than in WT and EV control plants (Figure 4a), demonstrating that the host-mediated gene silencing of Mj2G02 was effective. Four independent T2 generation transgenic RNAi lines and control lines were used for M. javanica infection assays. RT-PCR confirmed the expression of the RNAi cassette in transgenic plants before inoculation (Figure S2b). The results showed that the number of adult females per root in the four independent RNAi lines at 30 dpi was significantly reduced (37.5%–47.1%) compared with those in control plants (Figure 4b).
2.5 | Mj2G02-transgenic Arabidopsis plants exhibit enhanced susceptibility to M. javanica

To further verify the potential role of Mj2G02 in nematode parasitism, four independent T$_3$ homozygous Mj2G02-transgenic Arabidopsis lines were used for the nematode infection assay. RT-qPCR was used to confirm the expression of Mj2G02 in the four transgenic lines (Figure 5a). The transgenic plants showed an increase in lateral root growth compared with the WT plants, but the average root weight did not change obviously (Figure S3). The average number of adult females per gram root and per root system was increased in Mj2G02-transgenic lines compared with WT plants (Figure 5b,c). These results demonstrate that all four transgenic lines were significantly more susceptible to M. javanica infection than the control plants, suggesting a role of Mj2G02 in nematode parasitism.

2.6 | Mj2G02 suppresses Gpa2/RBP-1-triggered cell death

For the cell death suppression assay, we employed a coinfiltration Agrobacterium-mediated transient gene expression assay using N. benthamiana leaves. Agrobacterium cells carrying the Mj2G02Δsp construct were infiltrated into N. benthamiana leaves 24 hr prior to a second infiltration of Agrobacterium cells carrying Gpa2 and RBP-1. As negative controls, leaves were infiltrated with the EV
and buffer followed by Gpa2/RBP-1. After 5 days, Mj2G02 and the positive control GrCEP12 displayed the ability to suppress cell death mediated by Gpa2/RBP-1 (Figure 6a). The average percentages of necrosis were 12.5% and 8.3%, respectively, compared to 81.6%–94.1% in the negative controls (Figure 6b). Therefore, these results strongly suggest that Mj2G02 might play a role in suppressing plant cell death.

2.7 Mj2G02 interferes with plant JA signalling and decreases JA-Ile accumulation

Next, we analysed the differentially expressed genes (DEGs) in roots of WT and Mj2G02-transgenic Arabidopsis by RNA-Seq. A total of 19,535 genes with fragments per kilobase of transcript per million mapped reads (FPKM) ≥ 1 were identified (Table S2). Among these, 2,275 DEGs were upregulated and 1,398 DEGs were downregulated in the Mj2G02-transgenic line compared with those in WT plants (Figure S4). The complete list of the DEGs is provided in Table S3.

Gene Ontology (GO) analysis of the DEGs involved in biological processes in Arabidopsis was performed using Agrigo. The three most significantly enriched GO terms were secondary metabolic process, apoplast, and tetrapyrrole binding (Figure S5a). To further investigate the functional classification of the DEGs, the Kyoto Encyclopedia of Genes and Genomes (KEGG) ontology of DEGs was conducted using the KEGG automatic annotation server (KAAS). The result showed that these DEGs were primarily enriched in the metabolic pathways involving carbon metabolism, plant hormone signal transduction, and starch and sucrose metabolism (Figure S5b).

In the JA signalling pathway, multiple jasmonate ZIM-domain (JAZ) genes, including JAZ1, JAZ2, JAZ5, JAZ6, JAZ7, JAZ8, and JAZ10, were found to be significantly upregulated, while the JASMONATE RESISTANT 1 (JAR1) gene encoding a JA amino acid synthetase that conjugates isoleucine to JA was downregulated in the Mj2G02-transgenic plants compared with WT plants. Moreover, the JA-responsive genes MYC3, UPI, THI2.1, and WRKY75 also showed a significant decrease (Table 1). The transcription of these DEGs was further validated by RT-qPCR in three independent Mj2G02-transgenic lines (Figure 7a–c). All these genes showed a similar trend of transcription expression, supporting the RNA-Seq data.

The downregulation of JAR1 in the Mj2G02-transgenic plants prompted us to investigate the endogenous hormone jasmonoyl-isoleucine (JA-Ile) in Arabidopsis roots by high performance liquid chromatography mass spectrometry (HPLC-MS) analysis. The results indicated that the JA-Ile concentration was significantly lower in the two Mj2G02-transgenic Arabidopsis lines than in WT plants, with reductions of 41.8% and 41.5%, respectively (Table 2).

2.8 JAZ and JA-responsive genes respond to M. javanica infection

To investigate whether the upregulated JAZ genes and downregulated JA-responsive genes in Mj2G02-transgenic Arabidopsis lines were related to nematode parasitism, the expression levels of the
seven JAZ genes and four JA-responsive genes were analysed by RT-qPCR at 1, 2, and 5 days after infection (dai) by *M. javanica*. As a result, the expression levels of *UPI* and *WRKY75* were significantly increased at all three time points, compared with uninfected roots, the expression level of MYC3 was significantly increased at 1 and 2 dai, but not at 5 dai, and the expression levels of six JAZ genes

### TABLE 1  The relative expression levels of JAR1, JAZ, and JA-responsive genes in RNA-Seq data

| Gene ID   | Gene name | Gene description                                      | log$_2$FC | padj     |
|-----------|-----------|------------------------------------------------------|-----------|----------|
| 838501    | JAZ1      | Jasmonate-ZIM-domain protein 1                       | 1.349     | 8.14E−03 |
| 843834    | JAZ2      | TIFY domain/divergent CCT motif family protein       | 2.222     | 4.50E−06 |
| 838310    | JAZ5      | Jasmonate-ZIM-domain protein 5                       | 2.924     | 1.18E−08 |
| 843577    | JAZ6      | Jasmonate-ZIM-domain protein 6                       | 1.343     | 1.53E−02 |
| 818025    | JAZ7      | Jasmonate-ZIM-domain protein 7                       | 2.426     | 9.74E−06 |
| 839893    | JAZ8      | Jasmonate-ZIM-domain protein 8                       | 1.641     | 4.51E−04 |
| 831162    | JAZ10     | Jasmonate-ZIM-domain protein 10                      | 1.522     | 9.14E−03 |
| 819244    | JAR1      | Jasmonate resistant 1                                | −1.465    | 0.02     |
| 843558    | THI2.1    | Thionin 2.1                                          | −3.951    | 9.81E−05 |
| 834719    | MYC3      | Basic helix-loop-helix (bHLH) DNA-binding family protein | −1.234   | 0.01     |
| 834378    | UPI       | Serine protease inhibitor 2C potato inhibitor I-type family protein | −3.527   | 5.42E−09 |
| 831147    | WRKY75    | WRKY DNA-binding protein 75                          | −2.932    | 2.13E−11 |

The absolute value of log$_2$FC shows the expression fold changes of these differentially expressed genes in Mj2G02-transgenic *Arabidopsis* compared with wildtype plants. padj, adjusted or corrected p value.

### FIGURE 7  Confirmation of differentially expressed genes related to the jasmonic acid pathway in Mj2G02-transgenic *Arabidopsis* lines and wildtype (WT) plants by quantitative reverse transcription PCR. (a) The expression level of seven JAZ genes in Mj2G02-transgenic *Arabidopsis* lines showed a significant increase compared to those in WT plants. (b) The JASMONATE RESISTANT 1 (JAR1) gene expression level in Mj2G02-transgenic *Arabidopsis* lines showed a significant decrease compared to WT plants. (c) The mRNA expression level of four JA-responsive genes (MYC3, UPI, THI2.1, and WRKY75) in Mj2G02-transgenic *Arabidopsis* lines was significantly lower than those in WT plants. The *AtActin* gene (At1g49240) of *Arabidopsis* was used as the reference gene. Data are shown as the mean ± SD of three repeats. The experiments were performed two times with similar results, and three technical replicates for each reaction. *p < 0.05, **p < 0.01, Student’s t test. 2G1, 2G5, and 2G6 are three independent Mj2G02-transgenic *Arabidopsis* lines.
(JAZ1, JAZ5, JAZ6, JAZ7, JAZ8, and JAZ10) were significantly increased only at 1 dai, whereas JAZ2 and THI2.1 were significantly increased only at 2 dai (Figure 8). Our results suggest that all seven JAZ genes and four JA-responsive genes might be related to *M. javanica* parasitism.

**TABLE 2** Metabolic analysis of jasmonoyl-isoleucine (JA-Ile) in *Arabidopsis*

| *Arabidopsis* line | JA-Ile (ng/g FW root) |
|--------------------|-----------------------|
| Wildtype           | 30.23 ± 0.93a         |
| 2G1                | 17.58 ± 0.98b         |
| 2G5                | 17.68 ± 1.88b         |

2G1 and 2G5, two independent *Mj2G02*-transgenic *Arabidopsis* lines; FW, fresh weight. Data are presented as the mean ± SD (*n* = 3). Different lower case letters represent significant differences (Duncan’s multiple range test, *α* = 0.01).

3 | DISCUSSION

In this study, we cloned the *Mj2G02* gene from *M. javanica* and characterized its role in nematode parasitism. A BLAST search showed that various RKNs have genes with a high degree of homology to *Mj2G02*, while sequences with low homology occur in CNs and no homologs were found in other PPNs and free-living nematodes, suggesting that this gene may be conserved in *Meloidogyne* species.

The present study also demonstrated that the *Mj2G02* transcript is specifically present in the subventral oesophageal glands. It is believed that the two subventral gland cells of PPNs are essential secretory organs that participate in nematode penetration and migration in plant roots (Davis et al., 2000; Haegeman et al., 2012). Consistent with this notion, the *Mj2G02* developmental expression profile demonstrated that the highest expression level occurred in the pre-J2 stage and persisted at 2 dpi. This, together with the fact that *Mj2G02* possesses an N-terminal signal peptide that is
considered to aid protein translocation into the endoplasmic reticulum for secretion (Elling et al., 2007; Petersen et al., 2011), suggests that Mj2G02 is probably secreted during the stages of nematode invasion and migration in the roots. More importantly, Mj2G02-transformed Arabidopsis plants were more susceptible to *M. javanica* infection than controls and, conversely, in planta RNAi silencing of Mj2G02 significantly increased the plant resistance to *M. javanica*. Similarly, fewer galls, females, and egg masses were observed in transgenic *Arabidopsis* lines expressing Mi-msp2 (an Mj2G02 ortholog) dsRNA than control plants (Joshi et al., 2019). Taken together, these results strongly suggest that Mj2G02 plays a role in nematode invasion and the early stages of parasitism, promoting infection by *M. javanica*.

To resist the infection of pathogens, plants have evolved two layers of immunity: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI). In general, ETI is an accelerated and amplified PTI response, leading to an exacerbated cell death response at the infection site to prevent further development of the invading pathogen (Jones & Dangl, 2006). It is also suggested that the boundaries between PTI and ETI are not strictly distinct (Naveed et al., 2020; Thomma et al., 2011; Yuan et al., 2021). Some RKN effectors have been experimentally demonstrated to facilitate nematode parasitism by suppressing cell death (Chen et al., 2017; Niu et al., 2016; Shi et al., 2018; Zhuo et al., 2017). In the present study, using bioinformatic tools, we found that Mj2G02 has a predicted ShKT domain. Although the exact biological function of the ShKT domain remains unclear, previous reports have shown that this domain might be associated with immunosuppression. For example, *Toxocara canis*-secreted mucins (Doedens et al., 2001; Loukas et al., 2000) and the *M. incognita*-secreted effector Msp40 (Niu et al., 2016), both containing ShKT domains, are responsible for immune evasion and suppression of Bax-triggered cell death, respectively. Accordingly, an assay of cell death suppression by Mj2G02 was performed in *N. benthamiana* to explore whether Mj2G02 can suppress plant immunity. Indeed, the result showed that Mj2G02 could inhibit Gpa2/RBP-1-triggered cell death. Mj2G02 is predicted to contain two NLS motifs, and the transient expression assay confirmed its nuclear localization in plant cells. It has been previously reported that some nucleus-targeted effectors from PPNs, such as the *M. gramincola*-secreted effector MgGPP, *M. incognita*-secreted effector MiISE6, and *H. avenae*-secreted effector HaVAP2, can inhibit cell death (Chen et al., 2017; Luo et al., 2019; Shi et al., 2018). Thus, the molecular mechanisms of cell death suppression by nucleus-targeted effectors deserve further studies. Mi2G02, a homolog of Mj2G02, has been shown to be localized in the cytoplasm (Zhang et al., 2015); however, the Mi2G02 was fused to GUS protein, which might have resulted in the cytoplasmic localization. Taken together, these results suggest that secreted Mj2G02 might target the plant nucleus to suppress plant immunity and enhance *M. javanica* infection.

The phytohormone signalling network is required for plant immunity (Kazan & Lyons, 2014; Mine et al., 2018; Robert-Seilianiantz et al., 2011). JA and salicylic acid are considered the principal plant defence hormones and play an important role in fighting against pathogens, including RKNs (Gheysen & Mitchum, 2019; Gutjahr & Paszkowski, 2009; Molinari et al., 2014; Mur et al., 2006). Other phytohormones, such as abscisic acid and gibberellin, also play a role against RKNs, but mainly through crosstalk with the JA signalling pathway (Kvistert et al., 2017; Yimer et al., 2018). The present study showed that plant hormone signal transduction pathways including the JA signalling pathway were significantly enriched in Mj2G02-transformed plants. Transcriptome analysis and RT-qPCR showed that multiple JAZ genes were clearly upregulated, whereas JAR1 was significantly downregulated. It is believed that the upregulation of JAZ and the downregulation of JAR1 in the plant are beneficial for invading pathogens. For instance, previous studies showed that overexpression of JAZ7 enhanced the susceptibility of *Arabidopsis* to *Pseudomonas syringae* (Zhang et al., 2018), and the jar1-1 mutation increased the plant susceptibility to various pathogens (Berroccal-Lobo & Molina, 2004; Clarke et al., 2000; Ryu et al., 2004; Staswick et al., 1998). Evidence has emerged that the JA signalling is involved in resistance to RKNs in monocotyledons and dicotyledons (Fujimoto et al., 2011; Mendy et al., 2017; Nahar et al., 2011). For example, the expression of the *M. incognita*-secreted effector MiISE6 in *Arabidopsis* resulted in the upregulation of some JA genes and the downregulation of several JA-responsive marker genes, facilitating nematode parasitism in *Arabidopsis* (Shi et al., 2018); the tomato CO99 signalsome CSN4 and CSN5 interacted with JAZ7, a signalling component of the JA pathway, and played critical roles in JA-dependent basal defence against *M. incognita* (Shang et al., 2019); and *M. gramincola* infection in rice roots inhibited the accumulation of JA and JA-Ile, and foliar spraying of the strigolactone analog GR24 reduced JA and JA-Ile accumulation in rice roots, leading to a higher nematode infection (Lahari et al., 2019).

JAR1 is involved in the conjugation of JA to isoleucine for the synthesis of JA-Ile, which is considered to be the endogenous bioactive form of JA perceived by plants (Staswick & Tiryaki, 2004; Thines et al., 2007). Additionally, JAZ proteins can repress JA signalling via a regulatory negative feedback loop involving the transcription factor MYC2 (Chini et al., 2007; Thines et al., 2007). When the level of JA-Ile is low, the JAZ proteins recruit the NINJA/TOPLESS complex to repress the MYC2 activity, then repress in turn JA-responsive genes (Chico et al., 2008; Chini et al., 2007; Thines et al., 2007). Therefore, based on current knowledge, we speculate that Mj2G02 promotes nematode parasitism, probably modulating the JA signalling pathway via JAZ upregulation and JAR1 downregulation. This regulation results in a decrease of JA-Ile concentrations and suppression of JA-responsive genes. Consistent with this speculation, we found that the concentrations of JA-Ile were significantly lower in the Mj2G02-transgenic *Arabidopsis* lines than in WT plants. Moreover, the expression levels of four JA-responsive genes, MYC3, UPI, THI2.1, and WRKY75, which have been reported to be involved in plant defence against plant pathogens (Chen et al., 2020; Fernandez-Calvo et al., 2011; Kammerhofer et al., 2015; Laluk & Mengiste, 2011), were also significantly reduced in the Mj2G02-transgenic *Arabidopsis* lines.
In summary, we described a novel effector protein, Mj2G02, from *M. javanica* and investigated its role in *Arabidopsis* infection. Our experimental evidence suggests that *M. javanica* might secrete Mj2G02 into the plant cell nuclei during the early infection stages, reducing JA-Ile and promoting JAZ proteins to repress JA-responsive genes. JA-responsive gene repression impairs the plant immunity, increasing the plant susceptibility to *M. javanica* infection. Further studies of the interaction between the Mj2G02 effector and its receptor in plant cells may reveal the molecular mechanism controlling suppression of the JA signalling.

4 | EXPERIMENTAL PROCEDURES

4.1 | Nematode and plant materials

*M. javanica* was collected from towel gourd (*Luffa sp.*) in Guangxi, China, using a single egg mass, and maintained on tomato plants (*Solanum lycopersicum*) in a greenhouse at 25 °C under 16 hr light/8 hr dark (16/8 LD) conditions. Egg masses, pre-J2s, and parasitic stage nematodes were collected as previously described (Ding et al., 1998; Huang et al., 2005). *Arabidopsis thaliana* and *N. benthamiana* were cultivated at 25 °C in 16/8 LD cycles in a glasshouse.

4.2 | Gene cloning and sequence analysis

Total RNA was isolated from freshly hatched pre-J2s using the RNaprep Pure Micro Kit (TianGen Biotech). The cDNA was synthesized using the TransScript One-Step gDNA Removal and the cDNA Synthesis SuperMix kits (Transgen Biotech). The full-length cDNA sequence of Mj2G02 was amplified using the Mj2G02F and Mj2G02R primers designed based on the *M. incognita* sequence (Huang et al., 2003). All primers used in this study were synthesized by Tianyi Huiyuan Biotech and the full sequences are disclosed in Table S4.

The sequence of the predicted protein was used to identify homologous sequences by searches in the National Center for Biotechnology Information (NCBI) database, the WormBase database, and Nematode.net. The signal peptide was predicted using SignalP v. 4.0. Molecular mass was analysed using ProtParam. InterProScan was used to predict the putative conserved domains. The subcellular localization of effectors was predicted using the website PSORTII (http://psort.hgc.jp/form2.html) (Nakai & Horton, 1999).

4.3 | In situ hybridization

Approximately 10,000 pre-J2s of *M. javanica* were collected as described above. The 2G-ISHF and 2G-ISHR primers were employed to synthesize digoxigenin (DIG)-labelled sense and antisense cDNA probes based on the Mj2G02 fragment of 360–537 bp using a PCR DIG Probe Synthesis Kit (Roche Applied Science). The nematode sections were hybridized as previously described (Jaouannet et al., 2012) and the signals were detected by microscopy using an ECLIPSE Ni microscope (Nikon).

4.4 | Developmental expression analysis

Total RNA was isolated from approximately 200 *M. javanica* nematodes at different life stages as described above. The cDNA was then synthesized using the TransScript One-Step gDNA Removal and the cDNA Synthesis SuperMix kits (Transgen Biotech). RT-qPCR was performed using the qPCR2GF/qPCR2GR and qMj-ACT2-F/qMj-ACT2-R primer pairs for amplifying the Mj2G02 gene and the endogenous reference gene Mj-β-actin (accession no. AF532605), respectively. RT-qPCR was performed using the TransStart Tip Green qPCR SuperMix kit (Transgen Biotech) on a Dice Real-Time System thermal cycler (Takara). These experiments were repeated three times, with three technical replicates for each reaction. The relative changes in gene expression were calculated using the 2^−ΔΔt method (Livak & Schmittgen, 2001).

4.5 | Subcellular localization analysis

To construct the Mj2G02ΔΔsp:eGFP plasmid, the sequence of Mj2G02ΔΔsp was fused to the N-terminus of eGFP and cloned into pCambia 1305.1. eGFP alone was used as a control, and an auxin response factor 19 (ARF19) fused to the N-terminus of red fluorescent protein (RFP) was used as a nuclear marker. The constructs were purified using the HighPure Maxi Plasmid Kit following the manufacturer’s instructions (TianGen Biotech). The *Arabidopsis* root protoplast isolation and transformation were carried out as described previously (Yoo et al., 2007). Protoplasts were incubated in the dark at room temperature for c.48 hr and examined under an ECLIPSE Ni microscope. To verify the intact Mj2G02ΔΔsp-eGFP fusion protein, western blot was performed with anti-GFP antibody (Transgen Biotech) as described previously (Zhuo et al., 2017). The proteins were visualized using the Immobilon Western Chemiluminescent system with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Additionally, Mj2G02ΔΔsp:eGFP and eGFP plasmids were also transiently expressed in *N. benthamiana* leaves to analyse the subcellular localization as described previously (Chen et al., 2017).

4.6 | In planta RNAi

For the Mj2G02 silencing construct, the fragment of 109 to 405 bp within the Mj2G02 sequence was selected as the RNAi target. This sequence was confirmed to have no contiguous 21-nucleotide identical hits in other genes. Mj2G02ΔΔsp-405 was inserted into pMD-18T (Takara) in both sense and antisense orientations separated by a GUS intron. Then, the entire RNAi structure was inserted into pCambia 1305.1 to generate the plasmid expressing the hairpin dsRNA. The
recombinant plasmid was transformed into Agrobacterium tumefaciens EHA105, and an empty vector (EV) was used as the negative control. Next, the transgenic N. benthamiana plants were generated using the routine leaf disc method as described previously (Horsch et al., 1985). The RNAi transgenic lines were confirmed by RT-PCR using the GUS intron fragment as a target. To investigate RNAi efficiency, RNA was purified from 200 parasitic-stage nematodes collected from roots of 10 plants at 3 dpi. RT-qPCR was performed to determine the expression level of Mj2G02. Independent RT-qPCR experiments were performed three times.

4.7 | Generation of transgenic Arabidopsis plants

The coding sequence of Mj2G02 without the signal peptide was cloned into pCambia 1305.1 to generate the plasmid pCambia 1305:Mj2G02ΔSP. The overexpression plasmids were transformed into A. tumefaciens EHA105. Next, the transgenic Arabidopsis plants were generated using the floral dip method as previously described (Zhang et al., 2006). Transfornants were selected by hygromycin B on Murashige and Skoog (MS) medium, and T2 generation homozygous seeds were collected from T2 generation plants after selection by hygromycin B and were used in this study. The expression level of Mj2G02 in each transgenic line was determined by RT-qPCR. The Arabidopsis AtActin gene (At1g49240) was selected as an endogenous reference (Lin et al., 2016). The relative changes in gene expression were calculated using the 2−ΔΔCT method. Independent RT-qPCR experiments were performed three times.

4.8 | Infection assay

Fourteen-day-old Arabidopsis and tobacco plants were each inoculated with 150 M. javanica pre-J2s. At 30 dpi the roots were collected, washed, and stained by acid fuchsin (Naalden et al., 2018), and the number of M. javanica females was counted. Each experiment was performed three independent times. Statistical differences between treatments were calculated by Student’s t test using SAS v. 9.2 (SAS Institute).

4.9 | Cell death suppression

The coding sequence of Mj2G02ΔSP was cloned into pCambia 1305.1 to generate the pCambia 1305:Mj2G02ΔSP construct. The EV pCambia 1305 and pCambia 1305:GrCEP12 (Chronis et al., 2013) were used as the negative and positive control, respectively. The resistance/avirulence gene pair Gpa2/RBP-1 was used to induce cell death (Sacco et al., 2009). These constructs were separately transformed into A. tumefaciens EHA105 and then suspended in a buffer containing 10 mM MES (pH 5.5) and 200 μM acetosyringone. Subsequently, Agrobacterium cells carrying these constructs (OD600 = 0.5) were infiltrated into N. benthamiana leaves as described previously (Zhuo et al., 2017). After 24 hr, the same infiltration sites were injected with Agrobacterium cells carrying the constructs pCambia 1305:Gpa2 and pCambia 1305:RBP-1. The phenotypes of infiltrated N. benthamiana were photographed at 5 days after the last infiltration. The cell death phenotype was scored by an average necrosis percentage (Gilroy et al., 2011). This experiment was performed in triplicate.

4.10 | RNA-Seq

The Arabidopsis roots of 14-day-old WT plants and homozygous Mj2G02-transgenic plants (line 2G1) were used for the RNA-Seq analysis. The sequencing was performed using the Illumina HiSeq2500 system according to the manufacturer’s instructions, using two biological replicates. The raw reads from the sequencing run were filtered to remove adapter sequences, reads with more than 10% of unknown bases (N), and low-quality sequences to generate the final clean reads. The clean reads were mapped to the reference genome of Arabidopsis and subsequent analyses were performed.

The FPKM value of each gene was calculated based on the gene length and read counts mapped to this gene. Differential expression analysis was performed using the DESeq2 R v. 1.16.1 package. DEGs were selected based on criteria of an adjusted p value (padj) < 0.05 and absolute values of log2 fold change (log2FC) ≥ 1. GO annotation analysis and functional classification of DEGs were implemented using the GOSlim R package (Young et al., 2010). GO terms with padj < 0.05 were considered significantly enriched in DEGs. KEGG significant enrichment analyses from the KEGG database were conducted to identify the biological functions and related metabolic pathways in which these genes participate ( Kanehisa & Goto, 2000).

To validate the RNA-Seq results, RT-qPCR assays were performed using independently collected samples in the same developmental stage as those used for the RNA-Seq analysis.

4.11 | Arabidopsis gene expression analyses

For the Arabidopsis gene expression assays during a compatible interaction of M. javanica and Arabidopsis, seedlings were inoculated by M. javanica as described above. Arabidopsis roots infected by M. javanica and uninfected roots were sampled at 1, 2, and 5 dai. The total RNA of each sample was extracted and converted to cDNA as described above. RT-qPCR was performed to determine the expression changes of seven JAZ genes (JAZ1, JAZ2, JAZ5, JAZ6, JAZ7, JAZ8, and JAZ10) and four JA-responsive genes (MYC3, UPI, THI2.1, and WRKY75). The Arabidopsis AtActin gene (At1g49240) was selected as an endogenous reference (Lin et al., 2016). Independent RT-qPCR experiments were performed two times with three technical replicates. The gene expression levels of uninfected Arabidopsis roots were set to 1 to calculate the relative fold changes in M. javanica-infected roots. The relative changes in gene expression were calculated using the 2−ΔΔCT method (Livak & Schmittgen, 2001).
4.12 | Quantification of JA-Ile

For the analysis of the JA-Ile concentration, HPLC was performed on an EXIONLC System (SCIEX) as previously described (Simura et al., 2018). WT and Mj2G02-transgenic Arabidopsis were grown on 1/2 x MS medium, and root samples were collected from 14-day-old plants. A total of three biological replicates per treatment was used. The quantification of JA-Ile was confirmed by analysing serial dilutions of a standard mixture with multilevel calibration curves ($R^2 > 0.99$). The metabolite concentrations were determined relative to the corresponding internal standard.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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