B lack shank, a devastating disease in tobacco production worldwide, is caused by *Phytophthora parasitica* var. *nicotianae* Race 0 and injures the root, stem, and leaves of tobacco (*Nicotiana* spp.) plants at all growth stages, leading to leaf deterioration and even plant death, and the losses due to this disease have often been so high that tobacco can no longer be grown on affected farms (Gallup et al., 2006). Resistant resources to black shank in *Nicotiana tabacum* L. are scarce, with only Florida 301 and Beinhart 1000 usually mentioned. However, there are some resources with high-level resistance to black shank in wild *Nicotiana* species, such as *N. rustica* L., *N. longiflora* Cav., and *N. plumbaginifolia* Viv. (Litton et al., 1970; Li et al., 2006). The resistance of some of those wild species has been transferred to tobacco, with *N. plumbaginifolia* being one due to its high resistance to *P. parasitica* var. *nicotianae* race 0 (Drake et al., 2015). As early as the 1960s, the black shank resistance of *N. plumbaginifolia* was successfully transferred to flue-cured tobacco, and some cultivars, including PD468, NC2326, NC1071, and Coker 371-Gold, have been derived (Chaplin, 1962; Wang and Zhou 1995; Johnson et al., 2002a). A study conducted by Goins and Apple (1970) showed that black shank resistance in *Nicotiana tabacum* L. are scarce, with only Florida 301 and Beinhart 1000 usually mentioned. However, there are some resources with high-level resistance to black shank in wild *Nicotiana* species, such as *N. rustica* L., *N. longiflora* Cav., and *N. plumbaginifolia* Viv. (Litton et al., 1970; Li et al., 2006). The resistance of some of those wild species has been transferred to tobacco, with *N. plumbaginifolia* being one due to its high resistance to *P. parasitica* var. *nicotianae* race 0 (Drake et al., 2015). As early as the 1960s, the black shank resistance of *N. plumbaginifolia* was successfully transferred to flue-cured tobacco, and some cultivars, including PD468, NC2326, NC1071, and Coker 371-Gold, have been derived (Chaplin, 1962; Wang and Zhou 1995; Johnson et al., 2002a). A study conducted by Goins and Apple (1970) showed that black shank resistance in

**ABSTRACT**

Black shank is a devastating disease in tobacco production worldwide, and resistance to this disease in *Nicotiana plumbaginifolia* Viv. tobacco is controlled by the *Php* gene. The position of the *Php* gene on chromosomes of *N. plumbaginifolia* remains unclear. The tobacco line TP-1, which was derived from *N. tabacum* L. cv. Yunyan87 and *N. plumbaginifolia*, was used in the present study to help locate the *Php* gene on the *N. plumbaginifolia* chromosomes and understand the effects of the *N. plumbaginifolia* chromosome addition on the genomic expression of *N. tabacum*. An in vitro infection test verified that TP-1 was as resistant to *Phytophthora parasitica* var. *nicotianae* race 0 as *N. plumbaginifolia* and more resistant than Yunyan87. According to cytogenetic analysis, TP-1 was identified as a monosomic alien addition line and the alien chromosome was recognized as the ninth chromosome of *N. plumbaginifolia* through. Thus, the *P. parasitica* var. *nicotianae* race 0 resistance gene might be *Php* on chromosome 9 of *N. plumbaginifolia*. A superficial transcriptomic analysis showed that addition of the alien chromosome slightly affected the genomic expression of the host, with only 3.32% of differentially expressed genes (DEGs) identified between TP-1 and Yunyan87. According to Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, most (9/13) genes classified as involved in plant–pathogen reactions, particularly five serine/threonine-protein kinase genes, were upregulated in TP-1. It is worth noting that flavonoid biosynthesis may be greatly downregulated in TP-1: two genes were both downregulated in TP-1 according to gene ontology analysis, and all 20 DEGs in the flavonoid biosynthesis pathway were downregulated in TP-1 according to KEGG analysis.

**RESERCH**

**Resistance of Nicotiana tabacum to Phytophthora parasitica var. nicotianae Race 0 Is Enhanced by the Addition of N. plumbaginifolia Chromosome 9 with a Slight Effect on Host Genomic Expression**

Jiangbo Dang, Jinying Wang, Yao Yang, Wei Shang, Qigao Guo, and Guolu Liang*

College of Horticulture and Landscape, Southwest Univ., No. 2 Tiansheng Rd., Beibei District, Chongqing, China, 400715. Received 3 Jan. 2019. Accepted 8 July 2019. *Corresponding author (lianggl@swu.edu.cn). Assigned to Associate Editor Yiqun Weng.

Abbreviations: DEG, differentially expressed gene; FISH, fluorescence in situ hybridization; FPKM, fragments per kilobase of transcript per million mapped reads; GISH, genomic in situ hybridization; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LRR, leucine-rich repeat; MAAL, monosomic alien addition line; NCBI, National Center for Biotechnology Information; PCR, polymerase chain reaction; PMC, pollen mother cell; rDNA, ribosomal DNA; RAPD, random amplification polymorphic DNA.

Black shank, a devastating disease in tobacco production worldwide, is caused by *Phytophthora parasitica* var. *nicotianae* and injures the root, stem, and leaves of tobacco (*Nicotiana* spp.) plants at all growth stages, leading to leaf deterioration and even plant death, and the losses due to this disease have often been so high that tobacco can no longer be grown on affected farms (Gallup et al., 2006). Resistant resources to black shank in *Nicotiana tabacum* L. are scarce, with only Florida 301 and Beinhart 1000 usually mentioned. However, there are some resources with high-level resistance to black shank in wild *Nicotiana* species, such as *N. rustica* L., *N. longiflora* Cav., and *N. plumbaginifolia* Viv. (Litton et al., 1970; Li et al., 2006). The resistance of some of those wild species has been transferred to tobacco, with *N. plumbaginifolia* being one due to its high resistance to *P. parasitica* var. *nicotianae* race 0 (Drake et al., 2015).

As early as the 1960s, the black shank resistance of *N. plumbaginifolia* was successfully transferred to flue-cured tobacco, and some cultivars, including PD468, NC2326, NC1071, and Coker 371-Gold, have been derived (Chaplin, 1962; Wang and Zhou 1995; Johnson et al., 2002a). A study conducted by Goins and Apple (1970) showed that black shank resistance in
N. plumbaginifolia tobacco was controlled by a simple dominant factor (the Php gene). Complete linkage between 26 random amplification polymorphic DNA (RAPD) markers and the Php gene was confirmed in the K 326-BC5 generation by Johnson et al. (2002b). Johnson et al. (2002a) also studied the linkage distance among the Ph gene from Coker 371-Gold, Php gene from N. longiflora, and Php gene from N. plumbaginifolia and revealed that Ph and Php are 3 to 3.2 cM apart but that Ph and Php are only 0.45 cM apart. However, many details about the Php gene, such as the essence of the gene and its expression pattern in N. plumbaginifolia and tobacco, remain unknown. The position of a gene on a chromosome affects the expression of the gene (Spradling and Rubin 1983; Wilson et al., 1990), and this effect is named the position effect (Stankiewicz, 2006). Gene introgression is the main goal of distant hybridization. After introgression, the gene position and its genomic background are both altered, and the gene expression might change. Therefore, the chromosomal location of interspecific translocated genes is important for understanding the expression patterns of the genes in different hosts. However, until now, the position of the Php gene on the chromosomes of N. plumbaginifolia has been unclear. Only some RAPD markers have been found to be linked to Php in N. tabacum (Johnson et al., 2002b).

Monosomic alien addition lines (MAALs) can be used to locate genes on chromosomes. For example, Reyes et al. (1998) located eight isozyme loci on four chromosomes of rice using MAALs. Heneen et al. (2012) used Brassica rapa L.–B. oleracea L. MAALs to verify that the seed color is controlled by major genes on two B. oleracea chromosomes and minor genes on five other chromosomes of this species.

Monosomic alien addition lines are defined as lines generated by a species to which a chromosome from another species has been added and are important intermediate materials of interspecific hybridization. Such lines of Triticum (Makino 1976; Chen et al., 1994; Fu et al., 2013), Oryza (Jena and Khush 1989; Multani et al., 1994, 2003), Gossypium (Sarr et al., 2011; Chen et al., 2014; Wang et al., 2016), Brassica (Srinivasan et al., 1998; Heneen et al., 2012; Kang et al., 2014), Nicotiana (Lewis and Wernsman, 2001), and other plants (Reaumont-Ramos and Wricke 1992; Shigyo et al., 1998; Singh et al., 1998; Ali et al., 2001; Chen et al., 2004; Vu et al., 2012) have been produced and reported. Some of these MAALs have special characteristics. Until now, only a few studies have focused on hybrids of N. tabacum and N. plumbaginifolia and their derivative lines. Moav and Cameron (1960) reported genetic instability in a N. tabacum × N. plumbaginifolia hybrid. Oka and Niinomi (1961) observed the cytogenetic features of an allotrisomic plant derived from N. tabacum and N. plumbaginifolia.

Additionally, alien chromosome addition may lead to gene expression changes in response to genomic environment alterations. Wilson et al. (2008) used a mouse strain carrying human chromosome 21 to determine on a chromosomal scale whether interspecies differences in transcriptional regulation are primarily directed by human genetic sequences or the mouse nuclear environment. Cho et al. (2005) examined the transcript accumulation patterns in barley (Hordeum vulgare L.), wheat (Triticum aestivum L.), and barley–wheat chromosome addition lines to investigate the expression of barley transcripts in a wheat genetic background. Dong et al. (2018) reported the transcriptional and epigenetic adaptation of stably inherited alien maize chromosomes in two oat (Avena sativa L.)–maize (Zea mays L.) addition lines. Nonetheless, there have been no reports on the effects of alien chromosome addition on the genomic expression of the host.

Genomic in situ hybridization (GISH) has generally been used in interspecific hybridization, and MAALs can be easily identified using this technique (Schwarzacher et al., 1992; Shigyo et al., 1998). The ribosomal DNA (rDNA) has a tandem repetitive structure, and each cluster has ~70 repeated units of ~43 kb each; moreover, the rDNA loci on chromosomes are conserved (Kobayashi, 2014). Hence, rDNA sites can be detected by fluorescence in situ hybridization (FISH), named rDNA-FISH, which has been used for identifying chromosomes (Fukui et al., 1994). The RNA sequencing is currently a conventional method of genomic expression investigation that is widely used in genomic research and can detect global gene expression in an individual (Wang et al., 2009).

In this study, to help locate the Php gene on N. plumbaginifolia chromosomes, a P. parasitica var. nicotianae race 0 resistant N. tabacum cv. Yunyan87–N. plumbaginifolia resistant MAAL was identified by GISH and the N. plumbaginifolia chromosome in N. tabacum by rDNA-FISH. To understand the effects of N. plumbaginifolia chromosome addition on the genomic expression of N. tabacum, N. tabacum genomic expression in the MAAL was investigated by RNA sequencing.

MATERIALS AND METHODS

Plant Materials

TP-1 was obtained from the backcross progeny of an allopolyploid (2n = 5x = 58, Fig. 1) that was synthesized through hybridization between the octoploid N. tabacum cv. Yunyan87 (from Yunyan87 chromosome doubling, 2n = 8x = 96) and N. plumbaginifolia (2n = 2x = 20) and selected by a preliminary infection test. Its leaves showed the same level of in vitro resistance to P. parasitica var. nicotianae race 0 as those of N. plumbaginifolia. TP-1 was propagated by tissue culture and transplanted into the ground outdoors when there were at least two expanded leaves on the regenerated seedlings. Yunyan87 and N. plumbaginifolia were obtained from the Tobacco Research Institute of the Chinese Academy of Agricultural Sciences in Qingdao in Shandong, China, in 2010.
The lesion indexes of leaves were calculated using the formula lesion index = lesion diameter/leaf width, and the lesion indices of roots and stems were calculated using the formula lesion index = lesion length/total length.

**Mitotic Chromosome Preparation**

Mitotic chromosomes were prepared according to the method described by Burns (1982) with some modifications. Tender corolla base segments (~0.5 cm in length) of TP-1 and *N. plumbaginifolia* were separated, treated with 0.002 mol L\(^{-1}\) 8-hydroxyquinoline solution at room temperature for 4 h and fixed in Carnoy’s solution (methyl alcohol/acetic acid = 3:1) overnight. The segments were subsequently incubated in mixed enzyme solution containing 3% cellulose (SCR) and 0.3% pectinase (Yakult). After 1.5 h, the enzyme solution was removed, and the segments were gently rinsed. Carnoy’s solution was added, and the corolla segments were dispersed on slides with tweezers and dried over the flame of an alcohol burner.

**Genomic In Situ Hybridization**

Genomic in situ hybridization was performed on chromosomes of TP-1 using the method described by Brammer et al. (2013). The slides were treated with 20 mg mL\(^{-1}\) pepsin for 10 min at room temperature. The DNA of Yunyan87 and *N. plumbaginifolia* was extracted from the fresh leaves of the plants as described by Bindler et al. (2007). Genomic DNA of *N. plumbaginifolia* was used as the probe, and Yunyan87 DNA was used as blocking DNA. Probes were labeled by random priming using Biotin-High Prime (Roche). The final concentration of the probe in the hybridization mixture was 0.25 ng mL\(^{-1}\), and the concentration of the blocking DNA was 2.5 ng mL\(^{-1}\). After hybridization and elution, the chromosomes were incubated in avidin-rhodamine (Roche). The probe was directly detected under a fluorescent microscope (Olympus). Photographs were taken using a charge-coupled device camera.

**Meiotic Chromosome Preparation**

Tender anthers were collected during the meiotic phase and fixed in Carnoy’s solution overnight. The developmental stage of the anthers was determined using the squash technique (Koul and Nagpal, 2004), and anthers at the meiotic stage were selected. Meiotic chromosomes were prepared following the method reported by Dang et al. (2015).

**Analysis of 18S rDNA Loci Locations on TP-1 and *N. plumbaginifolia* Chromosomes by Fluorescence In Situ Hybridization**

We performed 18S rDNA-FISH on chromosomes of TP-1 and *N. plumbaginifolia*. The 18S rDNA fragments were amplified and labeled with digoxigenin via polymerase chain reaction (PCR) using a PCR DIG probe synthesis kit (Roche). The primers used in the labeling process were 5’-CTGAGAAACGGCTAC-CACA-3’ (forward) and 5’-CC CATCCCAAAGTCCAAC-3’ (reverse). The FISH procedure was similar to the GISH procedure described above. The probe concentration was 0.2 ng µL\(^{-1}\), and no blocking DNA was used. After hybridization, the slides were stained with anti-digoxigenin-fluorescein

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**Pathogen Culture and Preparation of Spore Suspension**

*Phytophthora parasitica* var. *nicotianae* race 0, which was separated from infected tobacco plants on a tobacco farm in Fengjie, Chongqing, China, was provided by Professor Li Zhenlun (Southwest University, Chongqing, China). The race of the isolate was determined according to an inoculation test and TTC (2,3,5-triphenyl tetrazolium chloride) reaction test (Wang et al., 1991; Li et al., 2011). The pathogen was cultured on oatmeal agar medium, and spores were induced in 0.1% KNO\(_3\) solution (Su et al., 2011).

**In Vitro Test of Resistance to *P. parasitica* var. *nicotianae* Race 0**

When the plants had five leaves, Yunyan87, *N. plumbaginifolia*, and TP-1 leaves were stabbed with a needle tip on the right side of the main vein, and the stems and roots of the three lines were cut on the lower ends. A 20-µL spore suspension (1 × 10\(^6\) spores mL\(^{-1}\)) was smeared onto the wounds. The leaves, stems, and roots were then incubated on humid filter paper in enameled dishes sealed by polyethylene film at 28°C under a fluorescent lamp at 250 µmol photons s\(^{-1}\) m\(^{-2}\) for a 12-h photoperiod. At least three replicates of every line were set. Lesion was defined as rot according to the symptom description of Gallup et al. (2006).
RNA Extraction and RNA-Seq

Total RNA from tender leaves was isolated from TP-1 and Yunyan87 lines at the bud stage. Leaves from at least three individual plants were pooled together to obtain one biological replicate and then ground in liquid N. The samples were sent to the Biomarker Biotechnology Company, where the RNA was extracted and libraries were constructed. Three biological replicates of 1 g of tissue powder were used to extract and purify RNA. A total amount of 1 μg of RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB) according to the manufacturer’s recommendations. Clustering of the index-coded samples was performed using a cBot Cluster Generation System with a TruSeq PE Cluster Kit v4-cBot-HS (Illumina), according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina NOVA-Seq, and paired-end reads were generated.

RNA-Seq Data Analysis

Clean reads were obtained by removing reads containing adaptor, reads containing ploy-N, and low-quality reads from raw data and were aligned to the genome of N. tabacum cv. TN90 (https://www.ncbi.nlm.nih.gov/genome/425) using TopHat (2.0.9) software (Kim et al., 2013). New genes were annotated based on the following databases: Nr (National Center for Biotechnology Information [NCBI] nonredundant protein sequences), Nt (NCBI nonredundant nucleotide sequences); Pfam (protein family), KOG/COG (clusters of orthologous groups of proteins); Swiss-Prot (a manually annotated and reviewed protein sequence database), KO (Kyoto Encyclopedia of Genes and Genomes [KEGG] orthologue database), and GO (gene ontology). A differential expression analysis of the two lines was performed using DESeq. Fragments per kilobase of transcription per million mapped reads (FPKM) values generated with the Cufflinks tool were used to measure the differences in gene expression levels between TP-1 and Yunyan87 (Trapnell et al., 2012). Differentially expressed genes were identified based on the following criteria: absolute value of log(2)-fold change > 2, and P value < 0.001 (Anders and Huber, 2010). The resulting P values were adjusted using the Benjamini and Hochberg’s approach to control for the false discovery rate. Gene ontology enrichment analysis of DEGs was implemented using the GOseq R packages based on Wallenius noncentral hypergeometric distribution (Young et al., 2010). KOBAS software (Mao et al., 2005) was used to test the statistical enrichment of the DEGs in KEGG pathways.

RESULTS

Resistance of TP-1 to P. parasitica var. nicotianae Race 0

The responses of N. plumbaginifolia, TP-1 and Yunyan87 were different (Fig. 2). Within 2 d after infection with P. parasitica var. nicotianae race 0, semitransparent lesions appeared on the leaves, roots and stems of Yunyan87. After 4 d, semitransparent spots on the leaf extended past the main vein to the other half of the leaf. On the stems and roots, the lesions extended more than halfway up the organs from the incision. Some white hyphae were even found on the surfaces of the leaves and stems at that time. After 10 d, the lesions extended all over the leaves, roots, and stems, and the leaves, roots, and stems completely decayed due to the pathogen. However, no semitranslucen lesions extending from the infected sites were found on the leaves, roots, and stems of N. plumbaginifolia and TP-1 throughout the 10-d observation period after in vitro infection with P. parasitica var. nicotianae race 0. Only chlorosis was found around the sites of infection on the leaves after 4 d, and some additional chlorosis was randomly found all over the leaves after 10 d. Nontransparent black lesions indicating infection by other fungi or bacteria were randomly found on the roots and stems of both plants.

These results indicated that the leaves, stems, and roots of TP-1 were more resistant to P. parasitica var. nicotianae race 0 compared with those of Yunyan87 and as resistant as those of N. plumbaginifolia.

Cytogenetic Characteristics of TP-1

The chromosome observations showed that TP-1 plants (Fig. 3A) had 49 chromosomes. Genomic in situ hybridization of mitotic chromosomes showed that the expected red signals, which were from N. plumbaginifolia genomic DNA probes, spread all over one chromosome only. That is to say, among the 49 chromosomes of TP-1, only one chromosome originated from N. plumbaginifolia. At the meiotic metaphase, one univalent and 24 bivalents were found in most (72.92% of) pollen mother cells (PMCs) (Fig. 3B). On average, 1.14 ± 0.38 trivalents were observed per PMC (103 PMCs in total). Zero to one micronucleus was detected at meiotic telophase I, and zero to two micronuclei were detected at meiotic telophase II.

18S rDNA Location on Chromosomes of TP-1 and N. plumbaginifolia

We performed 18S rDNA-FISH on the chromosomes of TP-1 and N. plumbaginifolia. TP-1 showed nine sites of 18S rDNA, with one located in the alien chromosome from N. plumbaginifolia (Fig. 3A). On the other hand, only two 18S rDNA sites were found on chromosomes of N. plumbaginifolia by 18S rDNA-FISH. Additionally, the 18S rDNA sites overlapped light-stained regions on the ends of the two chromosomes. The two chromosomes were identified as the ninth chromosomes of N. plumbaginifolia according to their length (Fig. 4A).

The configuration of the two ninth chromosomes was clearer when the chromosomes were more extended.
The unique mapped reads rate of TP-1 was 87.62 ± 0.95%, which was also lower than that of Yunyan87 (88.82 ± 0.27%) but did not reach statistical significance. All 41,248 genes were detected in TP-1 and Yunyan87. A total of 1371 DEGs (3.32% of all expressed genes) were found between the two lines (Fig. 5). There were 571 downregulated and 800 upregulated genes in TP-1. Gene ontology enrichment analysis showed that 796 of the 1371 DEGs (3.32% of all expressed genes) were found between the two lines (Fig. 5). There were 571 downregulated and 800 upregulated genes in TP-1.

Response of *N. tabacum* Genomic Expression to the Addition of *N. plumbaginifolia* Chromosome 9

To briefly evaluate the response of *N. tabacum* genomic expression to the addition of the *N. plumbaginifolia* chromosome 9, the leaf transcriptomes of TP-1 and Yunyan87 were roughly analyzed. The rate of the mapped reads of TP-1 was 90.79 ± 0.73%, which was lower than that of Yunyan87 (91.69 ± 0.15%), but there was no significant difference. The unique mapped reads rate of TP-1 was 87.62 ± 0.95%, which was also lower than that of Yunyan87 (88.82 ± 0.27%) but did not reach statistical significance. All 41,248 genes were detected in TP-1 and Yunyan87. A total of 1371 DEGs (3.32% of all expressed genes) were found between the two lines (Fig. 5). There were 571 downregulated and 800 upregulated genes in TP-1.

Gene ontology enrichment analysis showed that 796 of the 1371 DEGs were assigned to 7620 GO annotations, which are divided into three categories: cellular components, biological processes, and molecular functions. There were ≤7.69% genes found to be DEGs in each secondary category (Supplemental Table S1). In the
cellular components category (Supplemental Table S2),
there were 784 annotations, and DEGs were only anno-
tated among 188 GO terms. More than or equal to 10% of
the genes in only nine annotations were DEGs, and
50% of genes were DEGs in one annotation: peroxisomal
matrix and microbody lumen (2/4). Of 131 annotated
terms, ≥50% of DEGs were upregulated in TP-1. In the
biological processes category (Supplemental Table S3),
there were 2262 annotations, and DEGs were only anno-
tated in 547 GO terms. More than or equal to 10% of
the genes in only 123 annotations were DEGs, and no less
than 50% of genes were DEGs annotated in 29 GO terms.
Of 284 annotated terms, ≥50% of DEGs were upregulated
in TP-1. In the biological process category (Supplemental
Table S4), there were 4328 annotations, and DEGs were
only annotated in 1303 GO terms. No less than 10% of
the genes in 185 annotations were DEGs, and ≥50% of genes
were DEGs annotated in 31 GO terms. In 856 annotated
terms, no less than 50% DEGs were upregulated in TP-1.

Below, we focus on some interesting annotations
according to the GO analysis. There were no DEGs in
nicotine metabolism–related processes, and only 14 DEGs
were among 333 genes involved in photosynthesis. Only
one DEG was among 42 involved genes in C fixation. One
DEG among four genes was found involved in N fixation
biological processes, and ≤12.5% genes were DEGs
involved in N other N–related biological processes. Less
than or equal to 4.82% genes were DEGs involved genes
in plant hormone–related biological processes. Only two
genes were annotated in plant innate immune response
activating processes (GO: 0002220, GO: 0002429, GO:
0002752, and GO: 0002768), and one was downregulated
in TP-1. There were no DEGs in other pathogen–associ-
ated processes. Two genes were DEGs involved in positive
regulation of flavonoid biosynthetic process, and they
were upregulated in TP-1.

According to KEGG enrichment, 11,878 genes were
annotated in 101 different pathways, and only 229 DEGs
were involved (Supplemental Table S5). Among these
DEGs, 97 were upregulated and 132 downregulated in
TP-1. At least 10% of genes in six annotations were DEGs,
and at least 50% of genes were DEGs annotated in one KO

Fig. 3. Genomic in situ hybridization (GISH) and 18S ribosomal DNA (rDNA)–fluorescence in situ hybridization (FISH) of mitotic chromosomes of TP-1 and chromosomal behavior of TP-1 at meiosis metaphase. (A) Only one chromosome originated from N. plumbaginifolia in TP-1 (red); nine 18S rDNA sites (green) were found on nine chromosomes of TP-1, and one of them was found on the alien chromosome. (B) One univalent (arrow) and 24 bivalents were observed in one pollen mother cell of TP-1.

Fig. 4. The 18S ribosomal DNA (rDNA) sites on N. plumbaginifolia chromosomes and configuration of the ninth chromosome in TP-1 N. plumbaginifolia. (A) Only one pair of chromosomes was found in N. plumbaginifolia with 18S rDNA (green). (B) The ninth chromosomes were telocentric chromosomes, and the light-stained regions were on opposite ends of the centromeres.
term, up to 71.43% (5/7). Of 35 annotated terms, >50% of DEGs were upregulated in TP-1.

In pathways similar to the GO annotations mentioned above, only three DEGs were found among all 50 genes involved in nicotinate and nicotinamide metabolism. Only two DEGs were found among 137 involved genes in photosynthesis; only one DEG was found among 193 involved genes in C fixation. Overall, four DEGs were among 102 genes involved in N metabolism, 10 were among 679 genes involved in plant hormone signal transduction, 13 were among 422 genes involved in plant–pathogen interaction, and 20 were among 96 genes involved in the flavonoid biosynthesis pathway.

It is worth noting that 9 of 13 DEGs involved in plant–pathogen interactions were upregulated according to KEGG enrichment analysis, including five serine/threonine–protein kinase genes, Pto-interacting protein 1 isoform X1, the serine/threonine–protein kinase PBS1, leucine-rich repeat (LRR) receptor–like serine/threonine–protein kinase FLS2 (partial), LRR receptor–like serine/threonine–protein kinase FLS2, and LRR receptor–like serine/threonine–protein kinase GSO1 (Table 1). In particular, the FPKM of the serine/threonine–protein kinase PBS1 was 0 in Yunyan87 compared with 8.23 ± 1.24 in TP-1. These data indicated that the addition of chromosome 9 induced the upregulation of some host genes involved in plant–pathogen interactions.

All 20 DEGs in the flavonoid biosynthesis pathway were downregulated in TP-1 (Fig. 6), and most of these genes, such as the chalcone synthase gene, trans-cinnamate 4-monoxygenase gene, naringenin 3-dioxygenase gene, and flavonol synthase gene, play key roles in flavonoid biosynthesis. According to the transcriptome data, flavonoid biosynthesis was possibly greatly weakened in TP-1 compared with in Yunyan87.

Combining GO analysis and KEGG analysis, the addition of chromosome 9 of N. plumbaginifolia slightly affected gene expression in Yunyan 87. The effect on plant–pathogen interactions might be pivotal, and that on flavonoid biosynthesis may be great.

**DISCUSSION**

Genomic in situ hybridization has been used to identify MAALs in many plants (Sarr et al., 2011; Vu et al., 2012; Fu et al., 2013; Wang et al., 2016). Although this technique can recognize alien chromosomes, GISH cannot verify whether the host chromosomes represent a complete set. Thus, the behavior of meiotic chromosomes must also be investigated because an alien chromosome in a MAAL will usually form a univalent at the metaphase of meiosis, whereas the host chromosomes form bivalents. Before GISH was introduced, this phenomenon was often used as the main evidence to identify MAALs, and it has been used as supplemental evidence in more recent studies (Makino, 1976; Jena and Khush, 1989; Multani et al., 1994; Shigyo et al., 1998; Chen et al., 2004). In most TP-1 PMCs, 24 bivalents and one univalent were found at meiotic metaphase, which indicated that only one alien chromosome was present. Therefore,
Table 1. Differentially expressed genes (DEGs) classified in plant-pathogen interactions in Yunyan87 vs. TP-1 according to Kyoto Encyclopedia of Genes and Genomes (KEGG) classification

<table>
<thead>
<tr>
<th>Gene</th>
<th>log₁₀(FDR)†</th>
<th>log₂(FC)‡ (TP-1/Yunyan87)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene-responsive transcription factor 1</td>
<td>5.29</td>
<td>2.99</td>
</tr>
<tr>
<td>Chitin elicitor receptor kinase 1-like, partial</td>
<td>3.02</td>
<td>−2.65</td>
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<tr>
<td>Ethylene-responsive transcription factor 1</td>
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<td>2.99</td>
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<td>2.15</td>
</tr>
<tr>
<td>Probable calcium-binding protein CML18</td>
<td>4.35</td>
<td>−2.26</td>
</tr>
<tr>
<td>Probable calcium-binding protein CML23</td>
<td>3.66</td>
<td>−2.34</td>
</tr>
<tr>
<td>Respiratory burst oxidase homologue protein D-like isoform X1</td>
<td>3.34</td>
<td>3.59</td>
</tr>
<tr>
<td>Respiratory burst oxidase homologue protein E-like</td>
<td>4.74</td>
<td>−2.13</td>
</tr>
</tbody>
</table>

† FDR, false discovery rate.
‡ FC, fold change.
§ LRR, leucine-rich repeat.

TP-1 is a MAAL. In addition, the chromosome introduced from *N. plumbaginifolia* enhanced the resistance of the host Yunyan87 to *P. parasitica* var. *nicotianae* race 0. Moreover, the alien chromosome in TP-1 was identified as the ninth chromosome of *N. plumbaginifolia*, as reported by Mouras et al. (1986). Hence, we conclude that the ninth chromosome of *N. plumbaginifolia* carries the *P. parasitica* var. *nicotianae* race 0 resistance gene. The gene should be *Php*, but we cannot verify this based on the present results.

*Nicotiana plumbaginifolia* and *N. tabacum* belong to two different clades in *Nicotiana* (Lewis, 2011), suggesting the presence of genes specific to each. The mapped rate of TP-1 was lower than that of Yunyan87, which might be related to the expression of genes on the alien chromosome. Only 3.32% of *N. tabacum* genes were found to be differentially expressed between TP-1 and Yunyan87. Besides, according to GO enrichment, ≤10% of genes were DEGs in all 50 secondary categories; according to KEGG enrichment, ≤10% genes were DEGs in 94/101 annotations. These data confirm that the addition of *N. plumbaginifolia* chromosome 9 affected the gene expression of the host, but the effect was slight.

Serine/threonine-protein kinase genes have been identified as key genes of the plant innate immune system. These genes—*the tomato bacterial speck disease (caused by Pseudomonas syringae pv. tomato)* resistance gene *Pto* (Martin et al., 1993), the rice bacterial blight disease (*caused by Xanthomonas oryzae pv. oryzae* race 6) resistance gene *Xa21* (Song et al., 1995), the *Arabidopsis* flagellin perception gene *FLS2* (Gómez-Gómez et al., 2001), the *AavPphB* protein recognition gene *PBS1* (Swiderski and Innes, 2001), the stem rust (*caused by Puccinia graminis*) resistance gene *Rpg5* (Brueggeman et al., 2008) in barley, the stripe rust (*caused by Puccinia striformis*) resistance gene *Yr36* (Fu et al., 2009) and powdery mildew (*caused by Blumeria graminis*) resistance gene *Stpl-V* (Cao et al., 2011)—are involved in pathogen perception and stimulate plant resistance to bacterial and fungal pathogens according to their phosphorylation (Walker 1994; Jones and Dangl, 2006). A *PBS1*-like protein in rice (*OsPBL1*) is potentially involved in resistance to rice stripe disease caused by the *Rice stripe virus* (Lee and Kim, 2015). Furthermore, Gao et al. (2015) reported that the serine/threonine protein kinase gene *NrSTK* from *N. repanda* Wild. ex Lehms. enhances black shank resistance. The expression of these genes does not rely on pathogen induction. In TP-1, all five serine/threonine-protein kinases were upregulated. It appears that the addition of *N. plumbaginifolia* chromosome 9 enhanced the pathogen recognition capacity of *N. tabacum* cv. Yunyan87. However, it is unclear whether this was the cause of the enhanced resistance of Yunyan87 to *P. parasitica* var. *nicotianae* race 0. It is possible that this query can be answered when the essence of the *Php* gene and its expression pattern are uncovered in the future.

Flavonoids are important for fragrance and pigmentation formation in plants (Forkmann, 2003). Currently, leaves are the main product of tobacco. Fragrance is the principal determinant of tobacco leaf quality. Flavonoids have been considered to play major roles in the fragrance of tobacco leaves (Davis and Nielsen, 1999). All DEGs involved in flavonoid biosynthesis between Yunyan87 and TP-1 were downregulated in TP-1. Thus, fragrance was possibly decreased in TP-1 leaves, and leaf quality might be reduced.

Although the addition of chromosome 9 in tobacco might have negative effects on leaf quality, the MAAL TP-1 can be used in research on the black shank resistance gene and tobacco black shank breeding. Although the *Php* gene was transferred into flue-cured tobacco long ago,
Fig. 6. Differentially expressed genes (DEGs) involved in the flavonoid biosynthesis pathway. Downregulated DEGs in TP-1 are indicated in red.
there are genome components from at least two lines, *N. tabacum* '402' and Golden Wilt, in varieties that have the *Php* gene (Chaplin, 1962). Additionally, the chromosome segments transferred from *N. plumbaginifolia* have not been identified, but there may be some undesirable genes from *N. plumbaginifolia* that are closely linked to *Php*. Therefore, it is difficult to research the essence of the *Php* gene and its expression pattern. The chromosome loaded with the *Php* gene was confirmed in the present study and will be isolated and sequenced in the near future. In addition, introgression lines with a single *Php* gene and unmixed genomic background will be obtained by multiple backcrosses to Yunyan87 with the assistance of GISH and molecular markers. At that time, the essence of the *Php* gene and its expression pattern can be uncovered.

**CONCLUSION**

These results indicate that the *P. parasitica* var. *nicotianae* race 0 resistance gene is located on chromosome 9 of *N. plumbaginifolia*. The addition of *N. plumbaginifolia* chromosome 9 slightly affected *N. tabacum* genomic expression: the pathogen recognition capacity of *N. tabacum* cv. Yunyan87 might be enhanced, and flavonoid biosynthesis might be greatly weakened.

**Supplemental Material**

Supplemental material is available online for this article.

**Conflict of Interest**

The authors declare that there is no conflict of interest.

**Author Contributions**

J. Dang conceived the experiment, produced the materials used and performed the GISH, FISH, fertility analysis, and black shank resistance test. J. Dang, J. Wang, Y. Yang, and W. Shang carried out the transcriptomic analysis. Q. Guo and G. Liang contributed to the manuscript editing. All authors reviewed and approved this submission.

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**References**


Makino, T. 1976. Genetic studies on alien chromosome addition to a durum wheat. I. some characteristics of seven monosomic addition lines of *Aegilops unballulata* chromosomes. Genome 18:455–462. doi:10.1139/g76-055


