Tobacco transcription factor WRKY4 is a modulator of leaf development and disease resistance

X.-J. REN[1,2], W.-D. HUANG[1], W.-Z. LI[3] and D.-Q. YU[2]*

College of Agriculture and Biotechnology, China Agricultural University, Beijing 100094, P.R. China[1]
Xishuangbanna Tropical botanical garden, Chinese Academy of Sciences, Kunming 650223, Yunnan, P.R. China[2]
Institute of Tobacco Agricultural Research, Yunnan Tobacco Science Academy, Yuxi 653100, Yunnan, P.R. China[3]

Abstract

The role of tobacco transcription factor WRKY4 in leaf development and biotic stress tolerance was studied using RNAi suppressed transgenic plants. The leaves were more numerous and wider in *NtWRKY4* RNAi suppressed transgenic lines compared to the vector control, while the levels of miRNA166 and miRNA396 were reduced in suppressed lines. *NtWRKY4* expression was markedly induced in response to salicylic acid (SA), but not to abiotic stresses. When infected by tobacco mosaic virus (TMV), the leaves of the transgenic plants were more twisted and displayed a more obvious mosaic pattern compared to those of vector-transgenic plants. Less TMV viral RNA accumulated in vector-transformed plants than in transgenic plants. The results indicate that NtWRKY4 is involved in leaf morphogenesis and antiviral defense, which is seldom seen in WRKY family members.

Additional key words: antiviral defense, leaf morphogenesis, *Nicotiana tabacum* L., salicylic acid.

Introduction

The transcription factor WRKY protein family is defined by the presence of the conserved amino acid sequence WRKYGQK, along with either a Cys_2His_2 or $Cys_2HisCys$ zinc-finger-like motif at its N-terminus (Eulgem *et al.* 2000, Dong *et al.* 2003). Common to these proteins is a DNA-binding region of approximately 60 amino acids, which comprises the absolutely conserved sequence motif WRKY adjacent to a novel zinc-finger motif, and which is exclusive to plants (Ülker and Somssich 2004). The WRKY proteins regulate the expression of target genes by specifically binding to the (T)TGACC(A/T) W box sequence in their promoter regions (Yu *et al.* 2001).

A number of studies have shown that WRKY proteins have a regulatory function in plant defense response to pathogen infection. Pathogen infection or treatment with a pathogen elicitor, salicylic acid (SA) or H_2O_2, has been shown to induce rapid expression of WRKY genes from a number of plants (Chen and Chen 2000, Yoda *et al.* 2002, Takemoto *et al.* 2003, Vandenabeele *et al.* 2003, Zheng *et al.* 2006, Shimono *et al.* 2007, and Ramesh Sundar *et al.* 2008). In *Arabidopsis*, 49 out of 72 tested WRKY genes responded to bacterial infection or SA treatment (Dong *et al.* 2003). RNA interference (RNAi)-mediated knockdown of OsWRKY45 compromised benzothi-diazole-inducible resistance to blast disease (Shimono *et al.* 2007). Ectopic over-expression of WRKY33, on the other hand, increased resistance to two necrotrophic fungal pathogens. The susceptibility of WRKY33 over-expressing plants to *Pseudomonas syringae* was associated with reduced expression of the salicylate-regulated pathogenesis-related (*PR*)*1* gene (Zheng *et al.* 2006).

The study of the functions and networks of WRKY transcription factors in defense signaling has made rapid progress in recent years (Eulgem and Somssich 2007). For instance, several groups have reported the importance of AtWRKY70, which appears to affect the balance between signaling branches promoting SA-dependent and suppressing jasmonic acid (JA)-dependent responses (Li *et al.* 2004, 2006). Loss-of-function of AtWRKY70 rendered plants susceptible to the bacteria *Erwinia carotovora* and *P. syringae* as well as the fungi *Erysiphe*

cichoracearum and *Botrytis cinerea* (AbuQamar 2006, Li *et al.* 2006). Moreover, AtWRKY70 is required for both basal defense and full *R*-gene (*RPP4*)-mediated disease resistance against the oomycete *Hyaloperonospora parasitica* (Knoth *et al.* 2007). Similarly, a small clade (subgroup IIa) of *WRKY* genes, comprising *AtWRKY18*, *AtWRKY40* and *AtWRKY60,* plays important and partly redundant functions in regulating plant disease resistance (Xu *et al.* 2006). Several WRKY factors act as negative regulators of resistance. For instance, basal plant resistance, triggered by a virulent *P. syringae* strain, was enhanced in Atwrky7 and Atwrky11/Atwrky17 T-DNA insertion mutants (Journot-Catalino *et al.* 2006, Kim *et al.* 2006). However, little research has been published on the involvement of WRKY genes in TMV infection.

WRKY proteins also have important regulatory roles, for example in regulating plant response to abiotic stress or development and senescence (Robatzek and Somssich 2002, Hinderhofer and Zentgraf 2001, and Johnson *et al.* 2002). Suppression of AtWRKY75 expression through RNAi silencing resulted in early accumulation of anthocyanin, increased lateral root length and number, increased root hair number, and increased susceptibility to

P starvation (Devaiah *et al.* 2007). AtWRKY53 protein plays a regulatory role in the early events of leaf senescence (Hinderhofer and Zentgraf 2001). Legume WRKY proteins have been shown to regulate seed dormancy and drought tolerance (Pnueli *et al.* 2002), while the barley SUSIBA2 (WRKY) protein binds specifically to both SURE (sugar responsive) and the W-box in the promoter of *iso1* (*isoamylase*1) gene to regulate the sugar signaling pathway (Sun *et al.* 2003).

Ülker and Somssich (2004) identified more than 40 WRKY knockout lines but rarely observed phenotypic alterations under standard growth conditions. *AtWRKY44* (*TTG2*), the first *WRKY* gene whose function was unequivocally determined, plays a key role in trichome development, mucilage production and tannin synthesis in the seed coat (Johnson *et al.* 2002). WRKY factors most probably co-regulate other developmental programs such as senescence, but firm evidence to support this is scarce.

In the present study, we report an RNAi transgenic approach in tobacco to ascertain the biological role of the NtWRKY4 protein in leaf development and disease defense against viral infection.

Materials and methods

Tobacco (*Nicotiana tabacum* L. cv. Honghua dajinyuan) and its transgenic derivatives were grown in a greenhouse with a temperature 18 to 30 °C, with natural sunlight and humidity in pots with soil and a small quantity of humus.

Five-week-old seedlings were used for treatments. Halves of five just matured leaves from different plants were treated with SA (0.2 mM) or exposed to cold (4 °C), heat (42 °C), salinity (250 mM NaCl) and PEG (25 %) by floating on solution or water. Samples were harvested 0, 2, 4, 8 and 24 h after treatments, frozen in liquid nitrogen and then stored at -80 °C for further analysis.

Total RNA was isolated following a method of Logemann *et al.* (1987). For Northern blot analysis, total RNA (usually 20 µg) was separated on an agarose-formaldehyde gel and blotted onto nylon membranes following standard procedures (Sambrook *et al.* 1989). The membranes were hybridized with $(\alpha\text{-}^{32}\text{P})$-dATP-labeled gene-specific probes. Hybridization was performed in *PerfectHyb Plus* hybridization buffer (*Sigma*, St. Louis, USA) for 16 h at 68 °C. The membranes were then washed once for 10 min with 2× SSC buffer (3 M NaCl + 300 mM sodium citrate) and 0.5 % sodium dodecylsulphate (SDS), twice for 20 min with 0.5× SSC and 0.1 % SDS, then once for 20 min with 0.1× SSC and 0.1 % SDS at 68 °C. They were then exposed to X-ray films at -80 °C. ^{32}P-dATP ($>1.1 \times 10^{14}$ Bq mmol^{-1}) was obtained from *Beijing Furui Biotechnology Co.* (Beijing, China). All chemicals were obtained from *Shanghai Sangon Biotechnology Co.* (Shanghai, China) and *TaKaRa Biotechnology* (Dalian, China), including the

tobacco primers.

Fragments for the following probes were acquired by polymerase chain reaction (PCR) or reverse transcriptase (RT)-PCR: *Ntw4a*, *Ntw4b*, *PR1* and *PR2* were amplified by PCR, and the gene fragment of TMV coat protein (CP) was amplified from total RNA of TMV-infected tobacco by RT-PCR.. *Ntw4a*, *Ntw4b*, *PR1*, *PR2* and *TMV-CP* fragments were amplified using the following primers, respectively: *Ntw4a:* 5'-ATCAAGGATGCGAAGC AAA-3' and 5'-CATTCCAAACCAACTAGTGCT-3'; *Ntw4b*: 5'-GATGGATC TGTAATATTGACCGA-3' and 5'-TCCACTAAAGTTGAGGTTTCGT-3'; *PR1*: 5'-TCT CCTATAGTCATGGGATTTGTT-3' and 5'-ATGGAC GTAGGTCGTTTCAA-3'; *PR2*: 5'-TGTAGAGAAAGC TGGAGGACA-3' and 5'-TGTTACCATGCATTAATT AATTGAA-3'; *TMV-CP*: 5'-ACTCCATCTCAGTTCGT GTTCT-3' and 5'-TCCAGTTCCTCTGATCAATTCT-3'.

For RT-PCR analysis, the total RNA extraction protocol was the same as that for Northern blot analysis except the final step using pure sterile water to dissolve RNA. RNA was reverse-transcribed using a cDNA synthesis kit according to the manufacturer's protocol after being treated by RNase-free DNase. *Actin* cDNA was amplified in a 0.03 cm^3 reaction mixture by PCR with the following conditions: denaturation for 30 s at 94 °C, annealing for 30 s at 61.8 °C, and extension for 20 s at 72 °C for 24 cycles. The specific primer sequences for *actin* were 5'-TCCTGGAATTGCTGATAGGA-3' and 5'-TTAGAAGCATTTTCTGTGCACAA-3'. The PCR products were run on a 1.8 % agarose gel. *NtWRKY3* cDNA was amplified by denaturation for 35 s at 94 °C,

annealing for 35 s at 57 °C, and extension for 30 s at 72 °C for 26 cycles. The specific primer sequences for *NtWRKY3* were 5'-AGGACTTAGGAGAAGCTACTACG-3' and 5'-GGATTCCAACCTATGGCAG-3'. PCR product was run on 1.5 % agarose gel.

To generate the *NtWRKY4* RNAi plasmid, a 385 bp fragment of the *WRKY4* coding sequence was amplified using the primers 5'-ATCAAGGATGCGAAGCAAA-3' and 5'-CATTCCAAACCAACTAGTGCT-3'. The amplified fragment was initially cloned into pUCm-T plasmid, and then cloned into the binary double-stranded RNA vector, *pHannibal*, to produce *pHannibal-A* and *pHannibal-B* with the reversed repeated DNA. The two reversed repeated DNAs were transferred to pOCA30 from *pHannibal-B* by BamHI digestion. The plasmid pOCA30 has a CaMV 35S promoter.

Tobacco was transformed and regenerated according to a modified leaf-disk method (Horsch *et al.* 1985). *Agrobacterium tumefaciens* strain GV3101 mediated the process. Primary transgenic plants from independent tissue clones were transferred to soil in a greenhouse. To guarantee the transformed lines, 300 μg cm^{-3} kanamycin was mixed into the Murashige and Skoog (MS) medium for leaf disk culture up to the appearance of roots. T_1 progeny segregated in a ratio about 3:1 on this medium.

Tobacco mosaic virus (TMV) in 10 mM phosphate buffer, pH 7.0, was inoculated into a leaf blade of 6 to 9-week-old tobacco plants using carborundum as an abrasive. At first, infected leaf (1 g) was grinded in a mortar, and then diluted with 10 cm^3 of phosphate buffer. Then, the mixed TMV liquid was rubbed to the abaxial side of one leaf on the top.

Results and discussion

To characterize the loss-of-function phenotype conferred by gene *NtWRKY4*, we generated RNA interference (RNAi) transgenic tobacco lines by expressing an inverted-repeat sequence of a 385 bp *NtW4a* fragment of the WRKY4 cDNA from near the C-terminal end (Fig. 1*A*). The sequence of the *NtWRKY4* cDNA clone, which was isolated from a cDNA library of TMV-infected tobacco seedlings, confirmed the identity of this cDNA to be the same as cDNA clone AF193771 (Chen and Chen 2000). More than twenty independent transgenic lines were identified by morphology selection, and two of these, line 3 (moderate suppression) and line 12 (strong suppression), were selected for further analysis by RNA gel blot analysis (Fig. 1*D*). *NtWRKY4* RNAi transgenic T_1 progeny (second generation) were selected on MS medium with kanamycin before planting into pots. About a quarter appeared to be etiolated and stagnant-growing plants. The ratio of anti-kanamycin plants to etiolating plants in line 3 was 155:47 ≈ 3:1 and in line 12 it was 439:147 ≈ 3:1. This confirms the dominating genetic expression of RNAi and conforms to Mendel's law. PCR analysis with one primer from vector pOCA30 and one primer from the transformed

sequence indicated that the inverted-repeat sequence of 385 bp was inserted into the genome of the progeny (Fig. 1*C*). Another sequence near N-terminus of the WRKY4 cDNA was used as a probe in Northern blot to detect the expression level of *NtWRKY4* gene in RNAi transgenic lines (Fig. 1*D*). The expression level of the *NtWRKY4* gene was significantly reduced in the T1 transgenic lines. On the other hand, the expression of *NtWRKY3*, which is partly homologous with *NtWRKY4*, did not change (Fig. 1*B*).

The most obvious differences between the leaves of the progeny of the vector control and RNAi transgenic lines (6 to 9-week-old plants) were the wider leaves in RNAi plants, the leafstalks of which were shorter and the lamina wider than those of the vector control. The length/width ratio was significantly higher in the vector control (2.08 ± 0.18 compared with 1.62 ± 0.18 of line 3 and 1.75 ± 0.13 of line 12, $P < 0.05$). The difference appears more distinct in plants grown without additional fertilizer during the growing period. The RNAi transgenic plants have about two more leaves than the vector control plants (which have about nine leaves in totals without regarding the
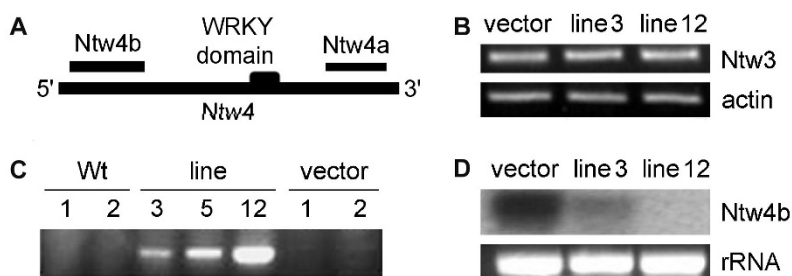

Fig. 1. Construction and identification of NtWRKY4 RNAi transgenic plants. *A* - Sketch map of the *NtWRKY4* DNA construct used in this study. *Ntw4a* is a short sequence in *NtWRKY4* cDNA adjacent to the C-terminus of the WRKY domain for transformation into plants. *Ntw4b* is a short sequence in *NtWRKY4* cDNA adjacent to the N-terminus of the WRKY domain for detection of *NtWRKY4* expression. *B* - RT-PCR analysis of *NtWRKY3* expression with tobacco actin as a control. *C* - PCR using one primer from pOCA30 and another from *Ntw4a*. Wt is wild-type. Vector is transgenic plant transformed with empty pOCA30 vector. *D* - Expression level of *NtWRKY4* by Northern blot analysis with *Ntw4b* as a probe under SA treatment. rRNA (20 μg total RNA) was used for RNA integrity.

cotyledon) and more leaves remain during the whole seedling period. Those changes in leaf morphology mentioned above might indicate that NtWRKY4 has an important role in leaf development. Apart from the expression level of endogenous *NtWRKY4* gene and wider leaves, no other obvious difference in phenotype was observed between plants from the vector control and the RNAi transgenic lines.

To confirm that *NtWRKY4* caused changes at the molecular level, we investigated the expressions of a few microRNAs that are reported to be related to leaf or shoot development. The result showed that the expression level of *MIR166* reduced sharply concomitant with the knock-down of *NtWRKY4*. *MIR396* also decreased in some way. *MIR319* did not appear to change among vector and transgenic lines (Fig. 2). Prigge (2005) reported that MIR166 was involved in the regulation of shoot meristem initiation and vascular development, therefore we can hypothesize that NtWRKY4 regulates shoot meristem initiation to influence the leaf number and regulates vascular development to influence the morphology of the leaf blade. The result is coincident with narrower leaves in transgenic plants over-expressing *miRNA396* (Liu *et al.* 2009).
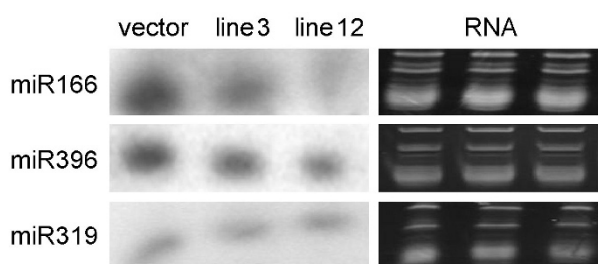

Fig. 2. Micro-RNA expression alteration in RNAi transgenic plants. Northern blot analysis using probes for miR166, miR396 and miR319. miRNAs are the smallest RNAs on the poly-acrylamide gel. Total RNA is 20 μg.

To understand the molecular function of NtWRKY4, we examined the expression pattern of the NtWRKY4 gene during treatment with SA, NaCl, PEG, low and high temperature by Northern blot analysis. The tobacco *WRKY4* gene was markedly induced by SA treatment in wild-type tobacco. However, there was little or no induction by the abiotic stresses mentioned (Fig. 3).

These results demonstrated that SA-induced WRKY genes are related to plant disease resistance and there is no difference in resistance to abiotic stresses between *NtWRKY4*-suppressed RNAi transgenic plants and vector control, therefore we focused on the role of NtWRKY4 in plant biotic resistance.

SA inhibits accumulation of TMV viral RNA and induces expression of *NtWRKY4* gene, which prompted us to test whether the *NtWRKY4* gene is involved in antiviral defense. The most notable symptom was the misshapen leaves that appeared after the transgenic plants were infected by TMV. After four weeks, the *NtWRKY4* RNAi transgenic plants had sharp misshapen leaves (forth to sixth leaf behind the incubated leaf), while the vector control plants showed little change. The leaves of the transgenic plants became abnormal, the blades narrowed at first and then became wider, and the leaf tip became sharp and narrow to form a cordiform shape (Fig. 4*A*). This change of leaf morphology might be a combined effect of TMV infection and the alteration of miRNAs level (Bazzini *et al.* 2007). We hypothesize that the alteration of some development-related miRNAs is caused by the suppression of *NtWRKY4* in this study.

The sixth to ninth leaf below the inoculated leaf had more obvious TMV mosaics compared to those of vector control plants. On the other hand, the malformed leaves gradually recovered and the leaf shapes almost recovered after ten weeks (Fig. 4*B*). The TMV mosaic is the most obvious difference compared to vector control plants 5 weeks after TMV infection (Fig. 4*C*). The mosaic is clear and dense on the leaves of RNAi transgenic line 3 and line 12 and also clear but sparse on the leaves of vector control plants. There was little difference in the mosaic after 10 weeks (Fig, 4*B*).

The most distorted leaves were the fourth to the sixth ones; therefore we selected the fourth leaf to detect TMV particle changes by Northern Blot analysis (Fig. 5*a*). TMV viral RNA in vector control leaves was the lowest compared to that of RNAi transgenic line 3 and line 12. The increase of TMV viral RNA, in combination with the morphological changes of TMV-infected leaves, indicates that vector control plants have stronger resistance to TMV infection and strongly inhibited TMV spread and/or the suppression of *NtWRKY4* reduced the resistance to TMV infection. These results suggest that *NtWRKY4* is involved in tobacco TMV-disease resistance.
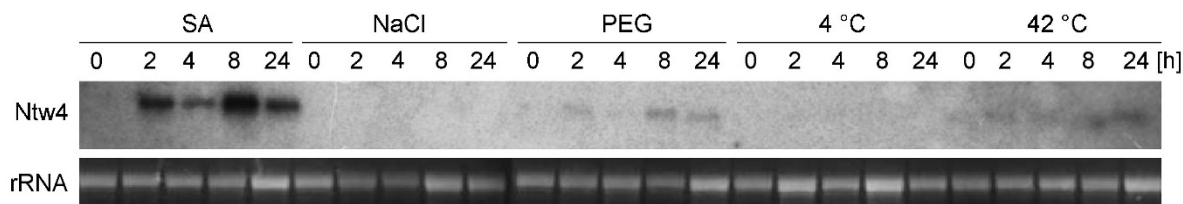

Fig. 3. Expression of *NtWRKY4* induced by different elicitors. 2 mM SA, 250 mM NaCl, 25 % PEG, 4 °C and 42 °C were used. Five newly matured leaves from five Wt plants in each treatment were floated on solution or water for the indicated duration. Ethidium bromide-stained rRNA under the blot demonstrates the RNA integrity (20 μg).
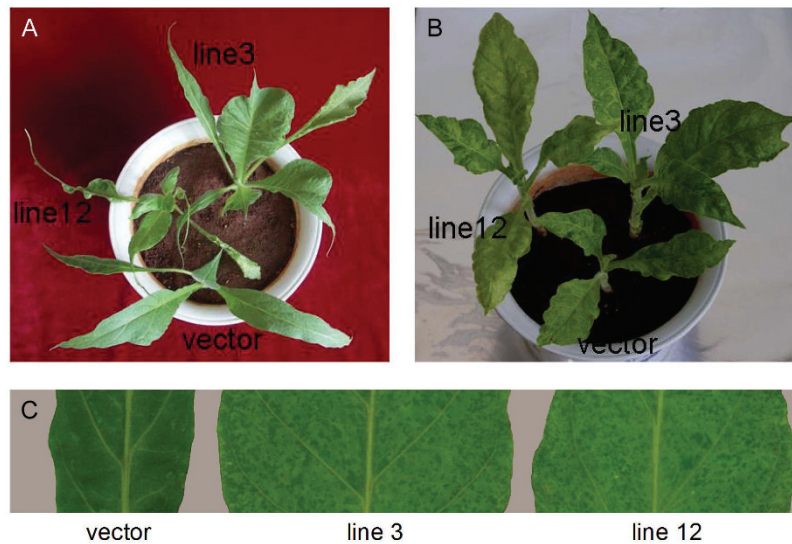
Fig. 4. Interaction of *NtWRKY4* and TMV in Ntwrky4 mutants. A - Shape changes of transgenic plants infected by TMV after four weeks on 5-week-old plants using carborundum as abrasive and TMV in 10 mM phosphate buffer. *B* - The leaves after the tenth leaf following the incubated one. The lower leaves have been removed. *C* - The middle part of the leaves infected by TMV after four weeks of growth. All the plants were grown in the open air in pots.
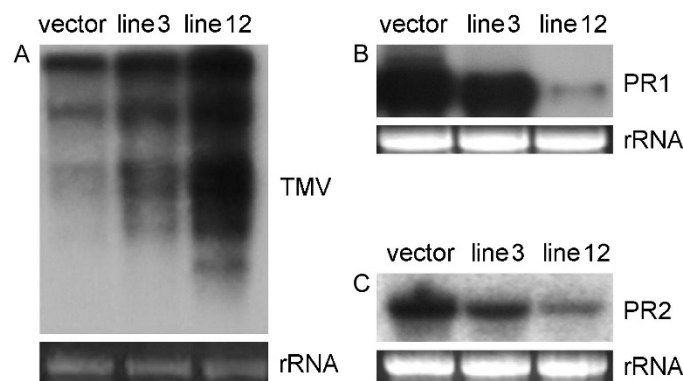

Fig. 5. Northern blot analysis of the levels of TMV, *PR1*, and *PR2* after treatment with TMV. *A* - TMV RNA content in the second leaves after the incubated ones of transgenic and vector control plants after seven days incubation (5 μg total RNA). *B* and *C* - the expression of *PR1* and *PR2*, respectively, after treatment with TMV for 10 d, in the second leaves behind the incubated ones of transgenic plants and those of the vector. Total RNA is 20 μg. Five leaves from five plants were gathered for each sample.

These results led us to examine the expression levels of pathogen-related genes *PR1* and *PR2*, which are reliable molecular markers of systemic acquired resistance (SAR) (Benedetti *et al*. 1998, Chevalier *et al*. 2008, Jaakola *et al*. 2008). Initial examination of the expression of *PR* genes revealed that RNAi transgenic line 3 and line 12 and vector control produced no detectable mRNA for *PR1* and *PR2* under normal growth conditions (data not shown). Under TMV-infection, the expression levels of *PR1* and *PR2* in the vector control transgenic line were strongly induced; however, not in transgenic lines (Fig. 5*B,C*). These results indicated that the *NtWRKY4* exerts its effects upstream of the *PR* genes in the TMV-resistant signaling pathway and the reduced resistance of RNAi transgenic plants to TMV, at the RNA level, might be due to inhibition of the signal transduction of *PRs* caused by

disruption of *NtWRKY4* gene expression.

In conclusion, many genes in the *WRKY* family have been shown to function in disease resistance (Eulgem and Somssich 2007) but few genes have been identified that they have developmental effects (Johnson *et al.* 2002). The most obvious phenotypic change in the present study is the wider leaves whose leafstalk are shorter and whose lamina are wider in the RNAi *NtWRKY4* transgenic plants compared to the vector control transgenic lines. Besides the wider leaves, the RNAi plants also have more leaves. NtWRKY4 probably has an important role in leaf initiation and leaf expansion (Barkoulas *et al.* 2007). The other tobacco WRKYs, which we have tested under the same procedure, for example NtWRKY1 and NtWRKY9, do not display this phenotype (data not shown). This study revealed that NtWRKY4 has some important function in

leaf development, although the mechanism is not clear. There is also a more obvious leaf morphological change in *NtWRKY4* RNAi transgenic plants when infected by TMV. The symptom is the misshapen leaves, especially the fourth to sixth leaf below the inoculated one. Recent research illustrated that TMV infection and TMV protein accumulation in tobacco was connected with changes in miRNA levels (Bazzini *et al.* 2007). In this study, the changes of leaf morphology in TMV-infected RNAi transgenic plants might be dependent on the expression of *NtWRKY4* gene, or be due to a synergetic effect of the miRNA levels and TMV accumulation; *i.e.*, NtWRKY4 controls the change and TMV boosts the change.

Besides being involved in leaf morphological

development, NtWRKY4 might have an important function in regulating responses to TMV-infection and its spreading. Viral RNA markedly increased in NtWRKY4 suppressed transgenic leaves (Fig. 5*A*). Meanwhile, the expression of *PR1* and *PR2*, which are induced by TMV-infection, was highly inhibited in *NtWRKY4* suppressed transgenic line (Fig. 5*B*,*C*). These results suggest that the reduced resistance of RNAi transgenic plants to TMV infection was due to suppression of the expression levels of *NtWRKY4*, *PR1* and *PR2* genes. So it is certain that the NtWRKY4 protein, which had a positive relationship with disease resistance to TMV, acts upstream of the PR proteins in the disease-resistance signaling pathway.

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