Expression of a WIPK-Activated Transcription Factor Results in Increase of Endogenous Salicylic Acid and Pathogen Resistance in Tobacco Plants

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NtWIF is a transcription factor activated upon phosphorylation by wound-induced protein kinase (WIPK) in tobacco plants. Transgenic tobacco plants overexpressing NtWIF exhibited constitutive accumulation of transcripts for pathogenesis-related genes, PR-1a and PR-2. Salicylic acid levels were 50-fold higher than those in wild-type plants. The levels of jasmonic acid and IAA did not significantly differ, while an increase of ABA upon wounding was delayed by 3 h in the transgenics. When challenged with tobacco mosaic virus, lesions developed faster and were smaller in the transgenic plants. The results suggest that NtWIF is likely to influence salicylic acid biosynthesis, being located downstream of WIPK.

**Keywords:** Hypersensitive response — Necrotic lesion — *Nicotiana tabacum* — Salicylic acid — Tobacco mosaic virus — Wound-induced protein kinase.

Plants respond to pathogens and physical injury by activating a specific set of genes, whose products function in healing of injury, restricting pathogens and preventing spread of damage. Transcript accumulation of these genes begins within an hour after recognition of stresses, continuing up to several days depending on their nature. Defense-related genes encoding, for example, pathogenesis-related (PR) proteins and proteinase inhibitors are expressed within several hours after wounding and pathogen attack. However, prior to expression of these terminal genes involved in the actual defense response, multiple signal transduction networks are considered to be switched on, leading to activation of the transcriptional machinery (Reymond et al. 2000, Ryan 2000).

Among early signaling events after wounding, activation of mitogen-activated protein kinases (MAPK) is notable (Zhang and Klessig 2001, Asai et al. 2002). In tobacco plants, wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK) are examples of such MAPKs, which have been best characterized to date (Seo et al. 1995, Seo et al. 1999; Zhang and Liu 2001). Phosphorylation activity of WIPK is induced a few minutes after wounding, and overexpressing lines show higher phosphorylation activity and higher jasmonic acid (JA) contents than controls (Seo et al. 1999).

The signaling pathways of WIPK and SIPK have been intensively studied. For example, expression of a constitutively active form of NtMEK2 (*Nicotiana tabacum* MAPK/ERK kinase 2), the upstream kinase of WIPK and SIPK, leads to activation of WIPK and SIPK, and induces hypersensitive response (HR)-like cell death (Yang et al. 2001). Similarly, activation of WIPK by a constitutively active MEK, NtMEK2DD, induces expression of several genes involved in pathogen responses, such as *PR-1a, PR-1b, PR-2* and *HMGR*. Activated WIPK also causes elevated expression of WRKY transcription factors (Kim and Zhang 2004), and SIPK has been shown specifically to phosphorylate WRKY1, resulting in induction of HR-like cell death (Menke et al. 2005).

Despite identification of upstream factors, downstream components of MAPKs have not necessarily been well characterized. Recently, we identified a target protein of WIPK from tobacco plants, and designated this as NtWIF (*N. tabacum* WIPK-interacting factor) (Yap et al. 2005). This 648 amino acid protein apparently possesses three distinct domains, the N-terminal DNA-binding B3 domain, the middle transactivation domain and the C-terminal WIPK-interacting domain. Upon binding to WIPK, the N-terminal region is specifically phosphorylated, resulting in transcriptional activation as tested with in planta luciferase reporter assays (Yap et al. 2005).

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Although these observations strongly suggest that NtWIF is a bona fide downstream element of WIPK, physiological analysis of challenged plants is necessary to provide evidence of direct relationships between the two. Here we report the production and characterization of transgenic tobacco plants expressing NtWIF, thereby showing that NtWIF positively controls salicylic acid (SA) levels, resulting in elevated resistance against tobacco mosaic virus (TMV).

After transformation, calli were propagated on selective medium and plants regenerated. Stem sections from T₀ plants were used to regenerate several plants per line. Among six lines initially selected, four lines (S4–S7) showed consistently high constitutive expression of NtWIF in both T₀ and T₁ generations (Fig. 1A). No visible alteration in phenotype or changes in development of these transgenic plants were observed, although they occasionally showed slightly lighter green leaves in comparison with control plants. In none of the plants was spontaneous lesion formation observed. To examine the correlation with wound and/or pathogen response, transcript accumulation for defense-related genes was initially examined by reverse transcription–PCR (RT–PCR). As representative genes, PR-1α and PR-2 were selected, and their transcripts were found to accumulate at constitutively high steady-state levels, with or without wounding in lines S4, S6 and S7 (Fig. 1B). This was in marked contrast to control plants, in which levels of both PR-1α and PR-2 transcripts were undetectable without stress, and temporarily accumulated to a lower level after wounding in comparison with transgenic lines (Fig. 1B).

The above results suggested that the defense-related signaling pathway was constitutively activated in transgenic plants. Subsequently, endogenous levels of wound-related signaling molecules including SA, which is a powerful inducer of PR-1α, were estimated using the line S7 as a representative of transgenic plants. The amounts of SA were approximately 0.1 and 6.8 nmol g⁻¹ FW⁻¹ of leaf tissues from unchallenged wild-type and S7 transgenic plants, respectively (Fig. 2A). This indicates the transgenic line constitutively to contain >50-fold higher SA levels than the control. When wounded, SA contents dynamically increased in the S7 plants up to 13 nmol h⁻¹ later. This increase was transient, showing a gradual decline to the basal level of about 4 nmol 3 h after wounding and thereafter (Fig. 2A). An increase of SA was also observed in wild-type plants, showing 0.5 nmol 1 h after wounding (Fig. 2A), although the absolute amounts were only 1/30 to 1/50 of those of the transgenic line. The increased level remained at about 0.4 nmol 3 h after wounding and later.

In contrast to SA, the level of JA was similarly 1 pmol g⁻¹ FW⁻¹ in both unchallenged wild-type and transgenic plants (Fig. 2B). Upon wounding, JA increased to 1 nmol in both cases after 1 h, and gradually declined to a level below 0.2 nmol by 12 h (Fig. 2B). A notable feature was that the decline was faster in the transgenic line than in the control, showing already the basal level of 0.3 nmol at 3 h in the former, as compared with >0.7 nmol in the latter at the same time point (Fig. 2B). The level of IAA did not significantly change after wounding in both wild-type and transgenic plants, with a basal concentration of approximately 30 pmol g⁻¹ FW⁻¹ (Fig. 2C). In the case of ABA, the basal level was about 0.32 nmol g⁻¹ FW⁻¹ in both plants without wounding (Fig. 2D). In wild-type plants, the level gradually increased up to 0.63 nmol g⁻¹ FW⁻¹ 6 h after wounding, and then declined to 0.46 nmol by 12 h. In transgenic plants, the level slightly declined at 3 h after wounding, and then increased in parallel with the control, continuing to increase up to 0.76 nmol by 12 h (Fig. 2D).

![Fig. 1 Expression profile. (A) Confirmation of transgenic lines. Total RNA was isolated from leaves of wild type (WT) or the indicated transgenic lines. A 10 µg aliquot per lane was fractionated by gel electrophoresis and, after transfer onto a nylon membrane, RNA was probed with a 32P-labeled NtWIF cDNA (NtWIF). Equal loading of sample RNA was confirmed with a 28S rRNA probe (28S). (B) Constitutive expression of PR genes. Leaves from the WT or the indicated transgenic lines (S4, S6 and S7) were harvested before (0) and the indicated time period after wounding, and total RNA was isolated. RT–PCR was performed with specific primers for PR-1α and PR-2.](https://academic.oup.com/pcp/article-abstract/47/8/1169/2329650)
This could be the result of a 3 h lag period at the onset of ABA increase in the transgenic plants.

The high SA level in the transgenic line suggested an altered response to pathogen attack. In order to assess this possibility, lesion development was evaluated upon TMV infection. Healthy leaves from wild-type and S7 plants were mechanically inoculated with TMV, and lesion formation was monitored 30 h after temperature shift. Results showed that, in S7 leaves, development of lesions appeared to be faster, including complete necrosis of the lesion, while their size was smaller in comparison with those in the wild-type controls (Fig. 3A). As transgenic plants contained elevated SA, effects of exogenously applied SA on wild-type plants were examined, to mimic the transgenic situation. Experimentally, 1 mM SA was applied daily to all leaves of wild-type plants for 4 d, and then TMV was inoculated and lesion development examined. The results showed a complete necrosis 30 h after temperature shift (Fig. 3A). Subsequently, lesion development was studied in detail over time. In wild-type plants, the first lesions were visible about 6–8 h after temperature shift. They gradually developed from the ‘early stage’, in which a dark ring occurs and tissue inside this ring is still green, to the ‘late stage’, in which the tissue inside the lesion consists of dead cells and is brownish in color (Fig. 3B, upper panel). Based on this measuring method, healthy leaves from wild-type plants, S7 plants and wild-type plants pre-treated with SA were inoculated with TMV and examined for lesion development at 24, 48 and 72 h after temperature shift (Fig. 3B, lower panel). In wild-type leaves, lesions were at the ‘early stage’ at 24 h, and developed to the ‘late stage’ 48 h later. After 72 h, all lesions completely matured, showing brown, large necrotic spots. In S7 leaves, a high percentage of lesions already developed to the ‘late stage’ at 24 h. Wild-type leaves pre-treated with SA showed a similar response to the S7 leaves. The differential lesion development was further examined at different time points after temperature shift (Fig. 3C). In wild-type leaves, all lesions were still in the early stage at 8 h, but about 24% of the lesions proceeded to the ‘late stage’ at 24 h, and >90% reached the late stage 48 h after the temperature shift (Fig. 3C). In contrast, about 26% of the lesions in transgenic plants were already in the ‘late stage’ 8 h after temperature shift, and 55% were at the late stage at the 24 h time point (Fig. 3C). In the case of wild-type plants that were pre-treated with SA, lesions developed rapidly, with 11% reaching the late stage at 8 h, and >90% at 24 h after temperature shift (Fig. 3C). Finally, lesion size at 72 h after temperature shift, when all lesions were at the ‘late stage’, was determined by measuring the average lesion diameter (Table 1). In wild-type plants, for average lesions, this was 3.93 ± 0.14 mm, whereas it was 1.67 ± 0.39 mm in transgenic plants. This was comparable with the 2.35 ± 0.19 mm noted for SA-treated wild-type plants. These results indicated that, in transgenic lines, lesions developed much faster and were smaller than in wild-type plants.

This report describes the construction and physiological analysis of transgenic tobacco plants constitutively expressing NtWIF, which is transcriptionally activated upon phosphorylation by WIPK under stress conditions. The transgenic plants were found to be phenotypically indistinguishable from wild-type plants, suggesting that the introduced gene was not detrimental for their development. However, the endogenous SA content was found...
to be about 50-fold higher than in the wild-type controls, even increasing a further 2-fold upon wounding. Because SA levels do not significantly change with wound stress in wild-type tobacco plants (Seo et al. 1995), the constitutively high SA content and its increase upon wounding in transgenic plants is unusual. Indeed, the observed high levels are similar to those induced by pathogen infection in wild-type plants, reaching up to 10 nmol (~1.4 μg g FW⁻¹ of leaf tissues (Yoda and Sano 2003). This implies that wounding mimics pathogen signaling in transgenic plants. In contrast, the contents of JA and IAA did not significantly differ between the transgenic and wild-type plants, suggesting a close relationship between SA metabolism and NtWIF function. This was confirmed by the observed pronounced resistance reaction against TMV, with transgenic plants developing smaller necrotic lesions at a faster rate than wild-type plants. The effect was apparently due to the elevated SA level, as exogenously applied SA resulted in similar promotion of lesion formation in wild-type plants. These results are consistent with the observation that transgenic tobacco plants expressing bacterial genes for SA synthesis overproduce SA and exhibit TMV resistance (Verberne et al. 2000). Since lesions usually develop as a result of the HR, it is highly conceivable that NtWIF positively regulates the HR. This idea is compatible with the finding that HR-like cell death is accelerated when WIPK is activated by its upstream kinase, NtMEK²DD (Liu et al. 2003).

Another notable finding is that the increase of the ABA concentration after wounding is delayed by 3 h in the transgenic lines, and a higher ABA content is measured 12 h after wounding, possibly reflecting a shift in the transient ABA increase. The significance of these differences is currently not clear, but involvement of ABA in wound and pathogen response has repeatedly been documented. For example, β-aminobutyric acid, which induces resistance against necrotrophic pathogens, was shown to depend on ABA signaling (Ton and Mauch-Mani 2004). In TMV-inoculated tobacco leaves, the ABA content was reported to increase 4-fold compared with untreated leaves 16 and 26 d after inoculation (Whenham et al. 1986). A cDNA microarray study in Arabidopsis showed many wound-responsive genes to be induced by ABA, and to be related to water stress (Delessert et al. 2004). Considering these observations, it is conceivable that the timing of the transient increase and the level of accumulation of ABA might be important to control the pathogen response properly through the MAPK cascade.

Overall, the present study suggested that NtWIF is involved in a process in which SA production is controlled, and that its overexpression might disturb cross-talk between wound and pathogen signaling pathways.

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**Fig. 3** Development of TMV-induced necrotic lesions. (A) Lesion formation. Healthy leaves from wild-type plants (WT), transgenic line S7 (#S7) or wild-type plants pre-treated with 1 mM salicylic acid (WT+SA) were detached, and were treated with tobacco mosaic virus (TMV), kept at 30°C for 2 d and then transferred to 20°C. Lesions were observed 30 h after temperature shift. (B) Development of lesions. Lesions of the early stage are characterized by dark ring-like structures with green tissue in the center, and lesions of the late stage are characterized by uniform brown circles consisting of necrotic tissue (upper panel). Examples of lesion development at the indicated time points upon TMV infection are shown in WT, (#S7) and WT+SA plants (lower panel). (C) Time course of lesion development. Lesion development was assessed as the percentage of late stage lesions in the total observed at the indicated time point. Samples were wild-type (open circles), transgenic S7 (filled circles) and wild-type plants pre-treated with 1 mM salicylic acid (open triangles) (see Table 1 for numbers of examined leaves and plants).
A critical question then arises as to the relationship between NtWIF and WIPK, which directly activates the former by phosphorylation (Yap et al. 2005). A previous study showed that co-suppression of WIPK resulted in high SA accumulation (Seo et al. 1995), apparently contradicting the present finding. Currently we have no clear explanation for this, but recent studies on Arabidopsis MPK4 are suggestive. When MPK4 was inactivated by transposon insertion, endogenous levels of SA were elevated, with a simultaneous increase in pathogen resistance (Petersen et al. 2000). A subsequent study identified an MPK4-interacting protein, MKS1, the overexpression of which again resulted in an increase of SA and pathogen resistance (Andreasson et al. 2005).

It was speculated that accumulation of unphosphorylated MKS1 exhibits a phenotype similar to that of plants lacking MPK4 (Andreasson et al. 2005). These observations are consistent with our results, although the phosphorylation status of overexpressed NtWIF in planta is currently not known. Whatever the mechanism may be, the MAPK cascade appears to be closely involved in adjusting the SA levels in both Arabidopsis and tobacco plants. Whether or not NtWIF functions directly in SA biosynthesis is not clear. It is tempting to speculate that it directly regulates genes involved in SA biosynthesis and turnover, although no such genes have so far been identified. In contrast, considering that SA levels sensitively change upon introduction of foreign genes, such as one encoding a small GTP-binding protein (Yoda and Sano 2003), the possibility remains that NtWIF indirectly regulates SA levels by, for example, modulating the network of wound/pathogen response pathways through activating other protein factor(s).

Table 1  Lesion size measurements

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of leaves</th>
<th>No. of plants</th>
<th>Total lesions</th>
<th>Average lesion size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>5</td>
<td>3</td>
<td>88</td>
<td>3.93 ± 0.14</td>
</tr>
<tr>
<td>Transgenic S7</td>
<td>8</td>
<td>4</td>
<td>364</td>
<td>1.67 ± 0.39</td>
</tr>
<tr>
<td>Wild type + SA</td>
<td>10</td>
<td>5</td>
<td>581</td>
<td>2.35 ± 0.19</td>
</tr>
</tbody>
</table>

*Examined samples were five leaves from three wild-type plants, eight leaves from four transgenic S7 plants (two leaves from each plant) and 10 leaves from five wild-type plants pre-treated with SA (two leaves from each plant).

Lesion size was measured 72 h after temperature shift. Average lesion size (mm) per leaf and number of lesions (in parentheses) were: 4.04 (20), 3.67 (12), 4.00 (12), 3.89 (18) and 4.04 (26) for wild type; 1.69 (54), 2.13 (54), 2.16 (92), 2.07 (8), 1.22 (17), 1.11 (94), 1.35 (39) and 1.6 for wild type; 1.69 (54), 2.13 (54), 2.16 (92), 2.07 (8), 1.22 (17), 1.11 (94), 1.35 (39) and 1.6 for wild type. The advantage of this evaluation system was that the observed values were independent of inoculated numbers of TMV per leaf. All series of experiments were performed with the three samples (WT + SA, and S7) in parallel to avoid fluctuation of lesion development, which is often affected by environmental conditions including humidity and light intensity. Lesion size was measured for inoculated leaves at the final mature stage of development. For the statistic analysis, the average lesion size per leaf was first counted using a minimum of five different leaves each from wild-type, S7 and SA-treated wild-type plants were evaluated. The advantage of this evaluation system was that the observed values were independent of inoculated numbers of TMV per leaf. All series of experiments were performed with the three samples (WT + SA, and S7) in parallel in triplicate to avoid fluctuation of lesion development, which is often affected by environmental conditions including humidity and light intensity. Lesion size was measured for inoculated leaves 72 h after temperature shift, when all lesions in all plants were visible. The advantage of this evaluation system was that the observed values were independent of inoculated numbers of TMV per leaf. All series of experiments were performed with the three samples (WT + SA, and S7) in parallel to avoid fluctuation of lesion development, which is often affected by environmental conditions including humidity and light intensity. Lesion size was measured for inoculated leaves 72 h after temperature shift, when all lesions in all plants were visible.

**Materials and Methods**

Transgenic tobacco plants (Nicotiana tabacum cv. Xanthi nc) overexpressing NtWIF were produced by replacing the GUS (β-glucuronidase) gene in the binary vector pBI121 (Clontech) with a full-length cDNA of NtWIF, ligated into the BarnHI–SacI-digested pBI121 vector and thus under the control of the cauliflower mosaic virus (CaMV) 35S promoter followed by the NOS terminator sequence. Leaf discs were transformed as described (Yap et al. 2002) and plantlets regenerated. Among six transformants initially obtained, four lines designated as S3–S7 were selected, and T0 and T1 plants were used for further experiments after confirmation of NtWIF expression by RNA blot hybridization. Both wild-type and transgenic plants were grown in a growth chamber at 23°C under a 14/10 h light/dark cycle. For wounding, intact leaves of 3-month-old plants were punched with a paper puncher and collected at appropriate time points. TMV inoculation was performed by mechanically inoculating detached leaves from about 2-month-old plants by rubbing with Carborundum, keeping them for 48 h at 30°C, at which temperature the resistance N gene does not function, allowing viruses to multiply, and then transferring to 20°C, to initiate the HR in a temperature- and humidity-controlled growth cabinet (Yap et al. 2002). Two to three leaves from each plant were assayed for one series of experiments, and in total at least three series were performed. Development of lesions was assessed 8, 24 and 48 h from the time of the temperature shift (t = 0). For determination of the percentage of fully developed lesions, at least five different leaves each from wild-type, S7 and SA-treated wild-type plants were evaluated. The advantage of this evaluation system was that the observed values were independent of inoculated numbers of TMV per leaf. All series of experiments were performed with the three samples (WT + SA, and S7) in parallel to avoid fluctuation of lesion development, which is often affected by environmental conditions including humidity and light intensity. Lesion size was measured for inoculated leaves 72 h after temperature shift, when all lesions in all plants were visible. The advantage of this evaluation system was that the observed values were independent of inoculated numbers of TMV per leaf. All series of experiments were performed with the three samples (WT + SA, and S7) in parallel to avoid fluctuation of lesion development, which is often affected by environmental conditions including humidity and light intensity. Lesion size was measured for inoculated leaves 72 h after temperature shift, when all lesions in all plants were visible.
and no detrimental effects on the plants were observed. For gene expression analysis, all harvested leaf samples were immediately frozen in liquid nitrogen. RT–PCR and RNA blot hybridization were performed as previously described (Yap et al. 2002, Yap et al. 2005), using total RNA isolated as described (Chomczynski and Sacchi 1987) with modifications. For hormone quantification, tobacco plants were wounded as described above, and leaves were harvested at 0, 1, 3, 6 and 12 h. Each leaf was split along the middle vein and one half was weighed and immediately soaked in 5 ml of 99% ethanol (p.a.) containing 30 pmol ml⁻¹ of each of the hormone standards ([²H]SA, [¹³C]JA, [²H]IAA, [²H]ABA acid, [²H]oxophytodienoic acid (OPDA); for details, see Müller et al. 2002). Samples were incubated at 50°C for 30 min and at 22°C for 16 h. The supernatants were transferred into reaction tubes and the solvent was removed in a vacuum concentrator (SpeedVac). Dried extract fractions were prepared for gas chromatography–mass spectrometry (GC–MS) analysis as described previously (Müller et al. 2002).

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