RESEARCH PAPER

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Effect of external and internal factors on the expression of reporter genes driven by the *N* resistance gene promoter

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Abbreviations: CaMV, cauliflower mosaic virus; CC domain, coiled coil domain; CCD, charge-coupled device; GUS, β glucuronidase; HR, hypersensitive response; LUC, luciferase; MS medium, Murashige and Skoog medium; R gene, resistance gene; SA, salicylic acid; TIR domain, Toll/interleukin receptor domain; TMV, Tobacco mosaic virus

The role of resistance (*R*) genes in plant pathogen interaction has been studied extensively due to its economical impact on agriculture. Interaction between *tobacco mosaic virus* (TMV) and the N protein from tobacco is one of the most widely used models to understand various aspects of pathogen resistance. The transcription activity governed by *N* gene promoter is one of the least understood elements of the model. In this study, the *N* gene promoter was cloned and fused with two different reporter genes, one encoding β-glucuronidase (N::GUS) and another, luciferase (N::LUC). Tobacco plants transformed with the N::GUS or N::LUC reporter constructs were screened for homozygosity and stable expression. Histochemical analysis of N::GUS tobacco plants revealed that the expression is organ specific and developmentally regulated. Whereas two week old plants expressed GUS in midveins only, 6-wk-old plants also expressed GUS in leaf lamella. Roots did not show GUS expression at any time during development. Experiments to address effects of external stress were performed using N::LUC tobacco plants. These experiments showed that *N* gene promoter expression was suppressed when plants were exposed to high but not low temperatures. Expression was also upregulated in response to TMV, but no changes were observed in plants treated with SA.

Introduction

The survival of an organism depends on its ability to counteract adverse environmental influence or stress. Plants are different in that they are unable to escape from their niche and have evolved the ability to resist many of these stresses. Among others, pathogen infection is one of the most relevant stresses due to its impact on agriculture. Plants inherit mechanisms to recognize and express resistance against specific pathogens. This results from molecular interactions between the pathogen and the host plant. The products of plant *R* genes can recognize pathogen derived elicitors or avirulence factors.[1,2] Upon recognition, R proteins trigger a signal, which results in localized cell death and formation of necrotic lesions, a reaction termed hypersensitive response.[1] Such an interaction is known as incompatible interaction. If the *R* gene is not present, the pathogen can infect and multiply in the host due to lack of resistance. Such an interaction is termed compatible interaction.[3]

The majority of R proteins contain an NBS domain and a C-terminal LRR domain. The LRR domain is known to play a role in the high specificity molecular interactions that are responsible for the recognition of a pathogen.[4] At the N-terminus, either a TIR domain or a coiled coil (CC) domain is present.

Tobacco infection with TMV has been widely employed as a model to understand the pathogen-host plant interaction. During a compatible interaction with tobacco, TMV causes mosaic-like symptoms. As for many other plant pathogens, there is a specific *R* gene for TMV in tobacco, the *N* gene, which imparts complete resistance against infection.[5] The *N* gene was named after the necrotic lesions on the leaf surface that are produced due to the hypersensitive response (HR).[6-8]

N gene belongs to the NBS-LRR class of *R* genes and contains an N-terminus that is similar to Toll and interleukin-1 receptors.[9-11] All the three domains, TIR, NBS and LRR are important for the function of N protein.[12] It is known that the Avirulence gene product recognized by the *N* gene is the replicase protein.[13] It is predicted that N protein forms a receptor complex and upon

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ligand binding a signal is transduced by the complex.[14] This signal results in HR against the TMV. There is a rapid burst of reactive oxygen species within ten minutes after TMV infection. This oxidative stress and the HR itself are salicylic acid (SA)-dependent; transgenic *NahG* plants expressing bacterial enzyme salicylate hydroxylase that degrade SA are not able to establish HR.[15]

The structure of the *N* gene coding sequence consists of five exons that encode a 131.4 kDa protein. Like several other *R* genes, *N* gene produces two transcripts as a result of alternative splicing at the third exon.[16] Transcription and translation result in a truncated protein of 75.3 kDa mass. A few previous reports reveal a function of alternative splicing in imparting resistance. However, it has been suggested that not only both transcripts are required for resistance, but also the specific ratio of shorter to longer transcript is important for the normal N function. This ratio, 28:1 (short:long) at the time of infection, changes to 1:23 6 h after infection and returns to 28:1 9 hr after infection. It is important to mention that previous work suggests that this change in the ratio occurs without a change in the transcription rate.[16] On the other hand, it should be noted that the work performed more recently did not confirm the change in the ratio of alternative transcripts and the role of this change in TMV resistance.[17]

Temperature plays a very important role in the interaction between TMV and the N protein. N protein is not able to trigger HR when the plant is grown at temperatures above 28°C and at this temperature TMV can systemically infect the plant. If a temperature-sensitized plant is brought back to a temperature below 28°C after infection, the *N* gene triggers global HR resulting in the death of the plant. Similar temperature-sensitive responses have been in past reported for cucumber mosaic virus infection of tomato[18] and *Arabidopsis* infection with *Pseudomonas syringae pv tomato* DC3000.[19] Although there might be many reasons for temperature sensitivity of plant immune responses, a recent report showed temperature-dependent changes in activity, stability and cellular localization of the SNC1 protein encoded by *Arabidopsis* NB-LRR type *R* gene.[20] The authors found a mutant in which a single point mutation, leading to a change from glutamic acid to lysine in the amino acid sequence, resulted in temperature-insensitive HR response. Introduction of a similar mutation into the N protein resulted in the same effect; tobacco plants were able to mount HR at high temperature.[20] These findings suggest that the N protein itself is temperature sensitive.

The role of transcriptional activity of the *N* gene in the resistance development is one of the least understood aspects. Earlier reports suggested that plants expressing both long and short versions of transcripts under the regulation of the *N* gene promoter are able to resist TMV. However, transgenic tobacco plants expressing both transcripts under the control of a 35S constitutive promoter are not able to mount HR.[16] In contrast, a different report showed that during a transient assay, the expression of both transcripts under the constitutive 35S promoter resulted in functional resistance to TMV.[21]

The native promoter and terminator was shown to be required for complete resistance again TMV. The study by Haque (2009) showed the existence of 20 nt sequence within *N* gene promoter (–269 to –250) that was essential and sufficient for activation by the 50 kDa helicase domain (p50).[22] The authors also demonstrated that activation of the *N* gene expression in transgenic plants carrying the N::GFP construct by p50 was not dependent upon interaction with N protein. They found however that the N protein alone is also able to stimulate the promoter activity in N::GFP plants. Presence of both, p50 and N proteins significantly increased GFP expression stemming from the *N* gene promoter, suggesting that these two proteins may still interact. This study was followed by the report that showed that activation of *N* gene transcription by p50 is actually N protein dependent; the authors showed that the critical region was located –290 to –271 upstream of the ATG and suggested the existence of two cis-regulatory elements, one being regulated by p50 alone (–269 to –250) and another regulated by p50/N protein pair (–290 to –271).[23]

The *N* gene expression was also shown to be regulated at the level of transcript. Researchers identified several microRNAs (miRNAs), including nta-miR6019 and nta-miR6020, that guide cleavage of *N* gene transcript.[24] Moreover, coexpression of *N* gene with these two miRNAs resulted in attenuation of TMV resistance, indicating the functionality of aforementioned miRNAs.

To understand the function of the *N* gene promoter region in more detail, we transformed tobacco plants of cultivar SR1 with the promoter:gene fusion consisting of a 4.1 kb region of the *N* gene promoter and either a LUC or GUS reporter gene. The expression of GUS driven by the *N* gene promoter was compared with plants expressing GUS constitutively (35S::GUS plants). The results indicate that *N* gene transcription is developmentally regulated. Experiments to characterize the effect of virus infection, temperature extremes and SA on reporter gene activity were also performed in N::LUC plants and results indicate that virus infection as well as high temperature affect *N* gene promoter driven expression and luciferase activity.

Results

Transformation and screening of plants. Tobacco cultivar SR1 lacking the *N* gene was used. This cultivar was used to avoid any possible influence of the presence of endogenous *N* gene on the *N* gene promoter driving the transgene expression. Also, since the N protein itself was shown to be able to regulate its own expression, we also wanted to remove this component. It is important to note that SR1 plants have all functional elements required for the tolerance to TMV except the N protein itself—transgenic SR1 plants expressing *N* gene resist TMV infection.[6] Regenerated SR1 transgenic plant lines were given a "SLUC" or "SGUS" prefix, standing for "SR1 LUC" or "SR1 GUS" expressing plants. For SLUC lines, out of 54 positive transformants from the T0 generation, 33 had a single locus insertion when tested for segregation in T1 generation (data not shown). Out of the 33 lines, 25 randomly selected homozygous lines were used for the expression analysis and two lines SLUC P2-1 and SLUC P6-1 were selected for further experiments (**Table S1**). For SGUS lines, out of 41 positive transformants, 22 had a single locus insertion and 20 homozygous lines were tested for the expression analysis

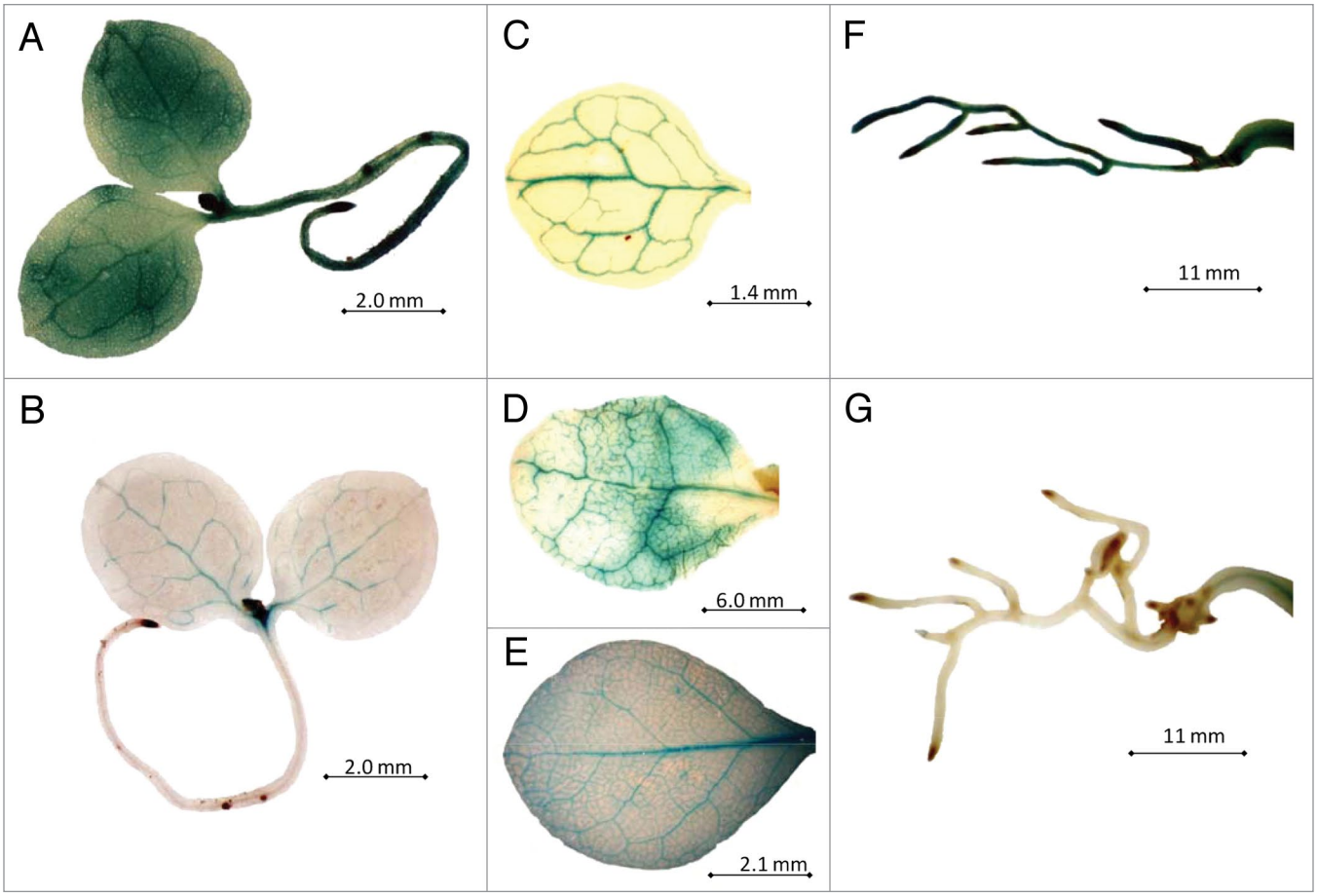

Figure 1. Expression of the *GUS* reporter gene in N::LUC and 35S::LUC tobacco plants. 35S::GUS (35SGUS 1-16) 2-wk-old tobacco seedling with expression in all tissues. (**A**) N::GUS (SGUS S12-4) 2-wk-old tobacco seedling with expression limited to vascular tissue of the cotyledon. (**B**) Single leaf of 2-wk-old N::GUS (SGUS S12-4) tobacco seedling. (**C**) Single leaf of 4-wk-old N::GUS (SGUS S12-4) tobacco seedling. (**D**) Single leaf of 6-wk-old N::GUS (SGUS S12-4) tobacco seedling. (**E**) Roots of the 4-wk-old 35S::GUS (35SGUS 1-16) plant. (**F**) Roots of the 4-wk-old N::GUS (SGUS S12-4) plant.

using histochemical staining. All 20 lines showed similar expression pattern and three lines, SGUS S1-9, SGUS S12-4 and SGUS S2-7, were selected for further expression pattern analysis.

Expression pattern analysis of N::GUS plants. Lines SGUS S1-9, SGUS S12-4 and SGUS S2-7 were used for analysis of expression pattern of the GUS reporter gene. As a control, two independent lines, 35S::GUS 1-9 and 35S::GUS 1-16, that have the GUS gene controlled by the CaMV 35S constitutive promoter, were used. Whole seedlings were harvested at 2, 4 and 6 wk of age.

In two-wk-old tobacco seedlings, the expression of GUS in N::GUS lines was limited to the midveins of the cotyledons, with no expression in the lamella. In contrast, the control 35S::GUS tobacco plants displayed uniform expression throughout the cotyledon (**Fig. 1A and B**). At 4 wk of age, the GUS activity began to spread into the lamella of tobacco true leaf and at about 6 wk of age there was uniform expression throughout the leaf (**Fig. 1C–E**). Primary roots from the N::GUS line did not show any GUS expression in the early seedling stages, whereas the 35S::GUS seedlings showed uniform expression in the roots (**Fig. 1A and B**). This absence of expression was found even in 4-wk-old N::GUS seedlings and beyond (**Fig. 1G**). In contrast, the expression of GUS in roots of control 35S::GUS plants was uniform from germination stage throughout maturity (data are shown for 4-wk-old plant, **Fig. 1F**).

The effect of high temperature on the LUC activity and expression. To assess the effect of high temperature on luciferase expression and activity, plants were shifted from 24°C to 45°C and analyzed at indicated time points.

Luciferase expression analysis by RT-PCR showed a significant decrease in luciferase expression starting at the 15 min time point (Student's t-test $p < 0.01$) and continuing at all later time points. To control for effects of changes in humidity, a set of plants was shifted outside the growth chamber. This affected luciferase transcript levels significantly at 15 and 60 min after the temperature shift (**Fig. 2A**).

Analysis of luciferase activity by CCD image analysis showed that there was a rapid increase in the intensity of luciferase activity within 15 min of incubation at 45°C. This increase eventually subsided with prolonged incubation. After 3 h, the expression pattern matched the intensity observed in control plants. After the 3 h time point, the intensity started decreasing and after 6 h there was no visible luciferase expression in the plant (**Fig. 2B, C**).

In an alternative assay, plants were incubated at 45°C and 24°C for 0–7 h time durations and luciferase activity was quantified

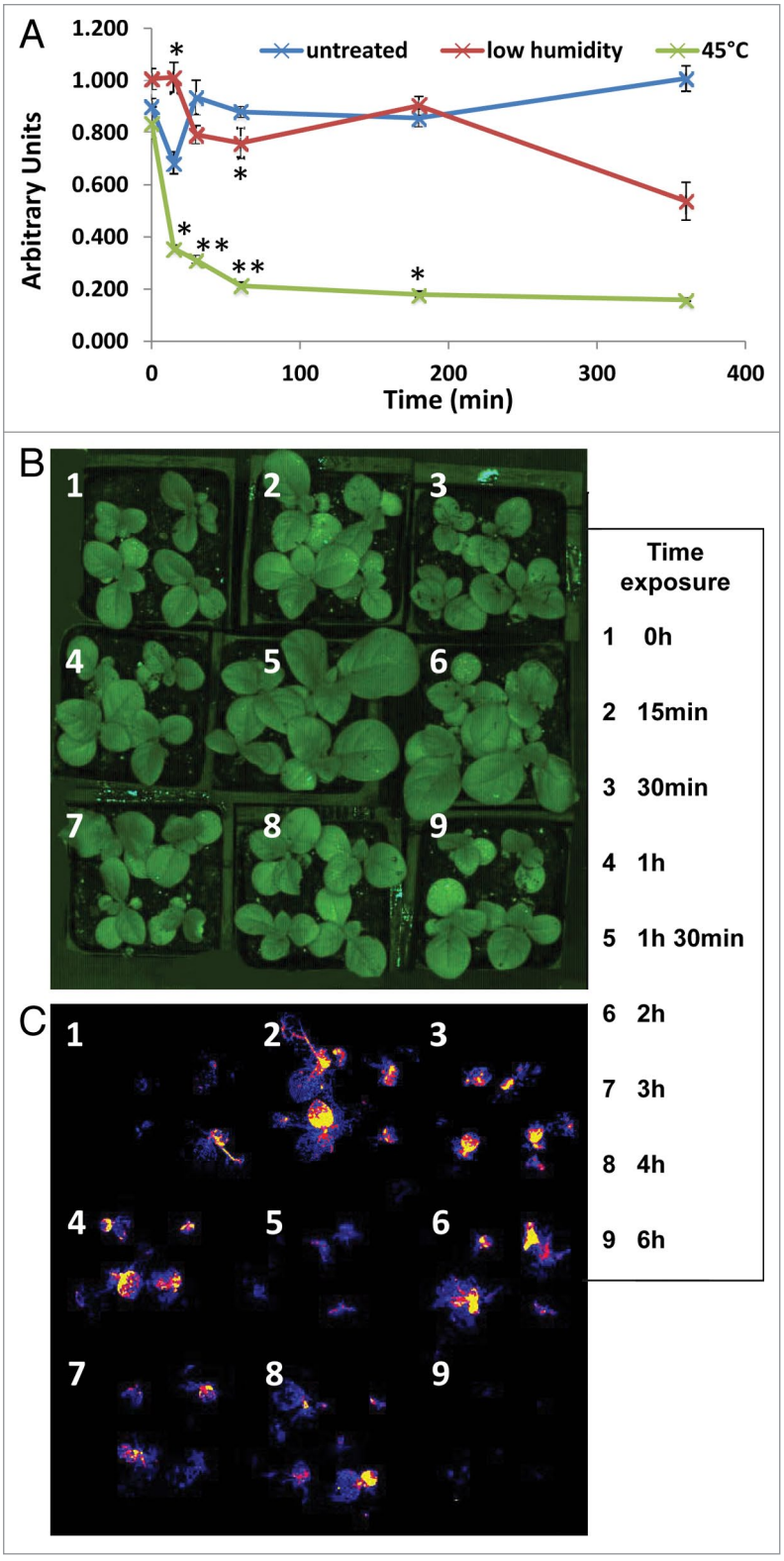

Figure 2. Luciferase activity and expression in SLUC P6-1 exposed to high temperature. (**A**) Relative luciferase expression levels as determined by RT-PCR. Data points represent an average of the expression levels in two different plants and two technical replicates per treatment determined at indicated time points and error bars show the error progression of the standard deviations from the technical replicates. (*Student's t-test $p < 0.05$; **Student's t-test $p < 0.01$). (**B**) Four tobacco SLUC P6-1 plants at each time point were analyzed after incubation at 45°C. Control image showing the placement of treated plants. (**C**) Dark image showing the difference in expression level.

using the luciferase quantification assay (**Table 1**). The comparison of LUC activity in plants exposed to 45°C or 24°C revealed two trends; initial increase in the activity, observed between 15 min to 3 h 30 min followed by a decrease in activity observed between 5 h and further time points, confirming the trend observed with the CCD camera images (**Fig. 2**). The comparison of values in control and high temperature treatment revealed that the means were significantly different in the samples prepared from heat-treated plants for all time (One-way ANOVA, $F_{15,63} = 2.181$, $p = 0.05$) (**Table S2**). Specifically, it was found that the 15 min, 30 min and 1 h treatments differed from the 0 h treatment (Student's t-test assuming equal variances, $p < 0.05$ in all cases). A further examination of the trend revealed that the values at the 15 min to 3 h 30 min were significantly different than the later time points from the 5 h mark and beyond (All Pairs Student's t-test, $p < 0.05$). At the same time, no statistically significant difference was found when similar comparison between time points was done for the control plants (One-way ANOVA, $F_{15,63} = 0.651$, $p = 0.870$) (**Table S2**). Another statistical analysis performed between 45°C and 24°C however, revealed no significant difference between these two groups of samples (One-way ANOVA, $F_{15,63} = 0.652$, $p = 0.816$, **Table 1**; **Table S2**).

The effect of low temperature on the LUC activity. When luciferase expression levels were checked by RT-PCR, no significant changes to luciferase expression were observed (**Fig. 3A**).

CCD image analysis revealed that there was high increase in the intensity of luciferase activity at 15 min after incubation at 4°C. This increase was reduced at later time points. The activity at 3 h post-treatment was similar between low and control temperatures. At the subsequent time points, LUC activity reduced further (**Fig. 3B and C**).

Alternatively, the LUC activity of luciferase was measured with luciferase quantification assay (**Table 2**). The averages observed within the 24°C control were found to be statistically identical (One-way ANOVA, $F_{15,63} = 0.359$, $p = 0.983$), while the averages within the cold treatment revealed statistically significant differences (One-way ANOVA, $F_{15,63} = 2.243$, $p = 0.0175$; **Table S3**). Another statistical treatment of data revealed no significant difference between normal temperature and cold treatment for all data points except 6 h 30 min (Paired Student's t-test, $p = 0.019$; **Table 2**; **Table S3**). The trend observed using the quantification assay showed certain similarities to the trend observed using CCD camera (**Fig. 3**). Also, certain similarities

between cold and heat treatments were found. In both cases, the increase of LUC activity was observed in plants exposed to either 4°C or 45°C from 15 min to 1 h 30 min after the exposure, although the data were significant only at 30 min time point ($p < 0.05$). Similarly, in both cases, the activity of LUC decreased at 5–6 h from the beginning of the exposure.

Effect of TMV on LUC activity. The effect of TMV infection on the activity of the *N* gene promoter was first analyzed by infecting detached leaves with 50 ng of TMV and monitoring luciferase activity using a CCD camera. The obtained images showed an increase of luciferase activity at the site of infection, noticeable at 30 min and most prominent at 2 h post infection (**Fig. S1** and **Movie S1**). No changes were observed in buffer treated leaves (data not shown).

The effect of TMV infection on the *N* gene promoter was also analyzed using the luciferase quantification assay. Leaves treated with either TMV or buffer were harvested at 0 h–7 h post-infection and the specific luciferase activity was measured. When we compared the values of individual treatment over time, the buffer treatment contained statistically identical averages (One-way ANOVA, $F_{15,63} = 0.889$, $p = 0.581$), as did the TMV treatment (One-way ANOVA, $F_{16,63} = 1.197$, $p = 0.306$) (**Table S4**). When values for particular time points were compared between buffer and virus treatment, luciferase activity was significantly higher for 1 h, 1 h 30 min, 2 h 30 min and 6 h 30 min time points in virus treated samples as compared with controls ($p < 0.05$ in all cases, **Table 3**; **Table S4**).

Effect of virus and high temperature on LUC activity. In this experiment, tobacco plants were grown at 24°C and shifted to 32°C at the age of 4 wk. TMV infection or a buffer treatment was performed at the age of 6 wk at 32°C. For CCD and RT-PCR analysis plants were shifted back to 24°C 24 h after infection and analyzed at indicated time points. For the luciferase quantification assay, plants were shifted to 24°C 7 d post infection; at this time infected plants started showing symptoms.

Quantitative real-time PCR analysis revealed a significant increase of luciferase transcripts 30 min after the plants were transferred to 24°C when comparing TMV vs. buffer treatment or no treatment (Student's t-test, $p < 0.01$) (**Fig. 4A**). This trend was also observed up to the 2 h time point, however it was statistically significant only 2 h after the temperature shift based on pairwise Student's t-test. Further, plants that were exposed to the buffer control (which involves wounding) showed significantly higher luciferase transcript levels when compared with untreated plants at 30 min after the temperature shift. A trend to increased luciferase expression following the temperature shift from 32°C to 24°C was observed in the control and buffer treated plants within the first 15 min, though not statistically significant.

CCD image analysis revealed that there was a clear induction of luciferase activity when plants were incubated for 2 h at 24°C after being transferred from 32°C. This induction was not observed in the subsequent time points. Furthermore, the area of high intensity corresponded to the likely area of TMV spread after a 2 h incubation period (**Fig. 4B**).

Luciferase activity of plants incubated at 24°C for 0 h to 7 h after the shift from 32°C to 24°C was further assayed using the luciferase quantification assay. The overall pattern for the specific activities indicated a trend as seen in CCD analysis (**Table 4**). In the comparison of values of treatments over time, it was found that the control plants had statistically identical means (One-way ANOVA, $F_{15,63} = 0.542$, $p = 0.903$), as did the TMV treatments (One-way ANOVA, $F_{15,63} = 0.779$, $p = 0.694$) (**Table S5**). Significant increase in LUC activity in virus-treated as compared with buffer-treated plants was observed for samples taken at 1 h 30 min and 2 h after shifting plants to 24°C (**Table 4**; **Table S5**).

The effect of salicylic acid on the LUC activity. The N::LUC plants were either sprayed with SA or buffer and the luciferase quantification assay was performed at different time points from 0 h to 7 h (**Table 5**; **Table S6**). The averages within the buffer treatment (One-way ANOVA, $F_{15,63} = 0.370$, $p = 0.981$) and the SA treatment (One-way ANOVA, $F_{15,63} = 0.995$, $p = 0.998$; **Table S6**) were found to be identical. The averages were quite uniform and second statistical analysis performed between SA- and buffer-treated samples revealed no significant differences (Student's t-test, $p > 0.1$, **Table 5**; **Table S6**).

Table 1. The effect of high temperature on LUC activity in tobacco SLUC P6-1 plants

Time	24°C (RLU), average	45°C (RLU), average	Change as compared with 24°C, %
0 h	12.55	12.85	+2.35
15 min	14.05	18.50	+31.64 ($p < 0.05$)
30 min	14.18	22.11	+55.92 ($p < 0.05$)
1 h	13.16	17.73	+34.79 ($p < 0.05$)
1 h 30 min	16.25	18.28	+12.46
2 h	16.18	19.24	+18.92
2 h 30 min	15.21	17.39	+14.33
3 h	14.23	16.48	+15.85
3 h 30 min	15.36	16.65	+7.78
4 h	14.35	16.12	+12.31
4 h 30 min	15.38	14.63	−4.88
5 h	16.78	11.95	−28.76
5 h 30 min	13.19	8.42	−36.19 ($p < 0.05$)
6 h	15.56	10.90	−29.93
6 h 30 min	17.93	11.05	−38.36 ($p < 0.05$)
7 h	16.16	12.00	−25.75

The tobacco N::LUC plants were incubated at either 24°C or 45°C. The table shows the difference in the averages of expression calculated from 4 plants per time point per treatment, done in triplicates with two technical repeats.

Discussion

Our results on development dependent GUS expression indicate that the *N* gene promoter is not as active in early developmental stages, suggesting that tobacco plants could be particularly sensitive to TMV at young age. Indeed, when we infected 2-, 4- and 6-wk-old plants with TMV, we found 2-wk-old plants to be the most sensitive to TMV, whereas 6-wk-old plants were the most resistant (**Fig. 5**). 2-wk-old plants infected with TMV had

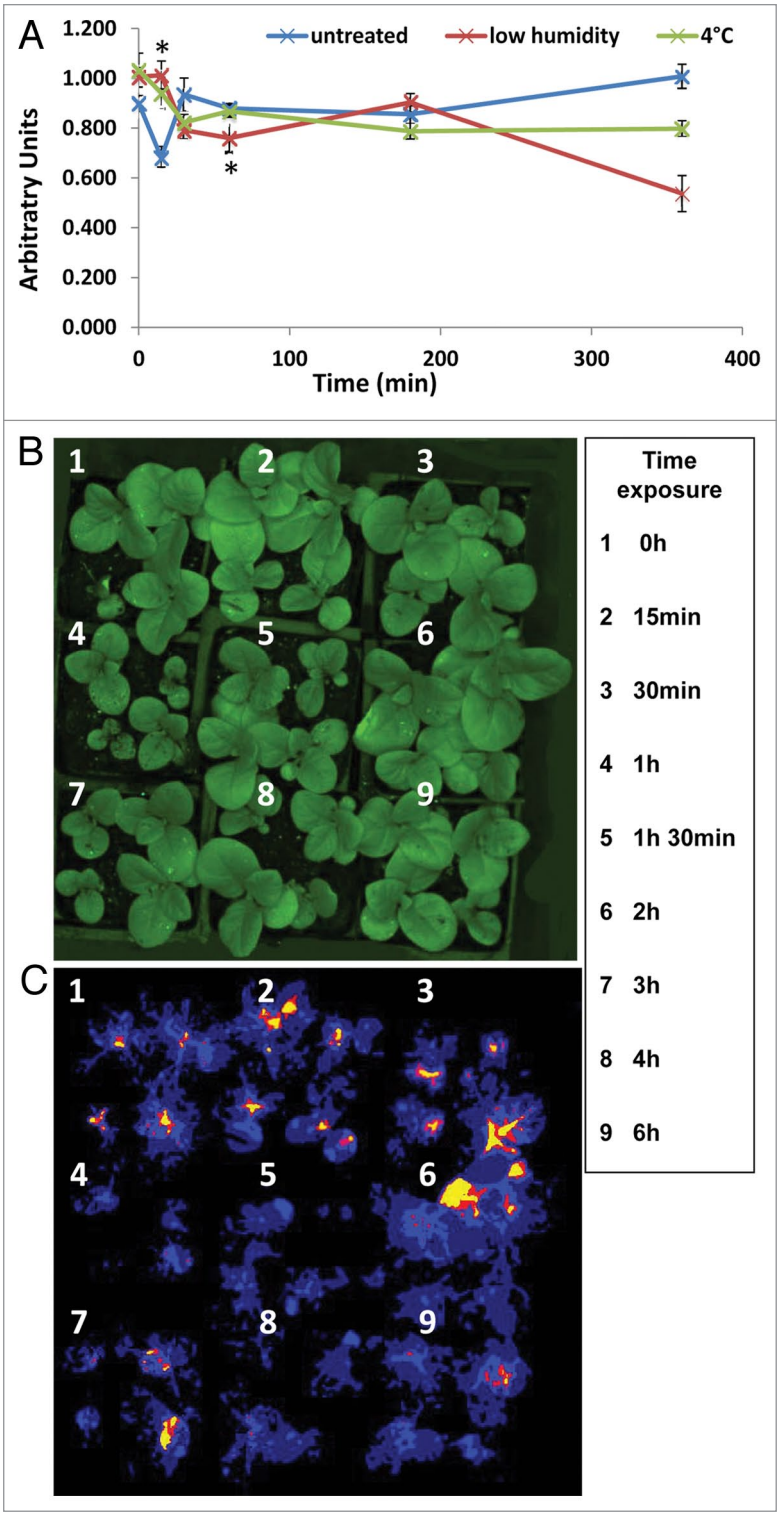

Figure 3. Luciferase activity and expression in SLUC P6-1 exposed to low temperature. (**A**) Relative luciferase expression levels as determined by RT-PCR. Data points represent an average of the expression levels in two different plants and two technical replicates per treatment determined at indicated time points and error bars show the error progression of the standard deviations from the technical replicates. (*Student's t-test $p < 0.05$). (**B**) Four SLUC P6-1 plants were analyzed for each time point. Control image shows the placement of treated plants. (**C**) Differences in expression level in the same plants in the dark image.

stunted growth and formed massive necroses. Infected leaves of 4-wk-old plants had numerous necrotic spots but were comparable in size to control or buffer-treated plants. Plants infected at 6 wk looked nearly completely normal, having only occasional necroses (**Fig. 5**). Similarly, it could be predicted that the roots would be more sensitive to TMV infection at any developmental stage. This, however, poses no threat to the plant, since roots are not a typical place for viral infection.

Further, it is very important to understand the role of the *N* gene promoter region in the interaction with TMV as it can regulate the level of transcription. When *N* promoter::reporter plants were inoculated with TMV and analyzed for reporter gene expression, an induction of luciferase expression corresponding to 1 h to 1 h 30 min after inoculation was observed. The CCD images showed localized areas of induction, which was likely due to the method of inoculation infecting a limited number of cells on the leaf. Further variation in this quantitative method could be due to some of the following reasons. Whole leaves were harvested for the infected tissue, resulting in the analysis of infected as well as uninfected cells; there is a high variability in the expression level in control plants, indicating high plant-to-plant variation. This may explain why the quantification assay failed to give more significant results. However, the induction as revealed by CCD analysis suggests that the transcription level of the *N* gene increases in the presence of TMV. As this increases early in the *N* gene mediated response to TMV, it can be suggested that this increase is important in the onset of resistance.

The *N* gene does not protect the tobacco plant against TMV at temperatures above 28°C. When the plants were inoculated with TMV at 32°C and then transferred to 24°C, they exhibited transcriptional induction of the luciferase reporter gene at the 30 min and 2 h time point. The CCD images showed a clear difference in the luciferase activity at the 2 h time point and the results from the luciferase quantification assay, although not significant, indicated a 1.46 fold increase in the reporter gene activity in TMV treated plants at the 2 h time point when compared with control plants. With the apparent variability in the reporter gene expression in plants, it is very likely that the induction is higher than was observed in the experiment. This early induction, as seen in the prior experiment, may be vital for *N* gene regulated resistance.

The increase in the reporter gene activity is most probably due to the increase in transcription, as observed in the RT-PCR analyses and further supported by the fact that no post transcriptional regulation for either luciferase or GUS has been reported in the literature. Similar transcriptional induction of a plant resistance gene has been observed for the incompatible interaction of Sunflower with *Plasmopara halstedii*, where

expression of the resistance gene was found to be significantly increased between 3 and 9 d post infection—coinciding with the peak of the hypersensitive response.[25]

Such an induction of *N* promoter dependent transcription in response to TMV infection suggests that a high amount of N protein might help an efficient response to the infection. This prediction contradicts the previous report that there is no change in the transcription level of *N* gene following TMV infection.[16] However, this report suggests that the *N* gene regulated by the 35S promoter and terminator imparts resistance. The transcription level of such a construct would be higher than the native promoter, as 35S is a constitutive promoter. This indicates that N protein is functional when present in high amounts. More experiments are needed to analyze the changes in accumulation of *N* gene transcripts or N protein.

Our observations suggest that increasing temperature suppresses *N* gene promoter driven expression. Therefore the initial increase in luciferase activity as observed by CCD analysis and luciferase quantification might be due to altered cell and protein metabolism. However the decrease in luciferase activity observed at later time points does seem to correlate with decreased *N* promoter dependent expression. Such suppression of *N* gene promoter dependent transcription might also explain why plants that are infected with TMV at temperatures higher than 28°C are not able to resist the infection. Future experiments using constitutive promoters driving LUC gene expression should be able to identify whether temperature has any effect on the metabolism of the luciferase protein or luciferase activity.

If at high temperature there was less N protein activity, it was logical to assume that this would result in less efficient response to TMV. Hence, the results predict that if the plants are incubated at 32°C for more than 6 h, they will not be able to resist pathogen infection and systemic spread. Furthermore, the results also predict that if the plant would be infected in the initial 1 h of incubation at 32°C, it would able to form necrotic lesions and suppress systemic infection as at this time point plants likely had sufficient transcriptional activity at *N* gene to actively mount the resistance response. Unlike exposure to high temperatures, exposure to low temperatures does not affect *N* gene promoter expression. Therefore, the prediction would be that plants infected with TMV at low temperatures should be capable of mounting resistance.

Further, as revealed by the luciferase quantification assay, external application of SA does not interfere with the LUC activity. It can be suggested that there is no influence of SA on the *N* gene promoter region. Indeed, it was shown that SA has no influence on accumulation of two transcript variants of the *N* gene.[24] Report from Levy et al. (2004) indicates an increase in *N* gene transcription after 72 h, in inoculated as well as non-inoculated leaves.[15] This increase in transcription may be due to influence of SA accumulation. However, the result suggests that there is no positive feedback mechanism for regulating *R* genes activity by SA accumulation. This is also in line with the observation of Radwan et al. (2005), that expression of a sunflower resistance gene that conveys resistance to downy mildew is not induced by SA.[25] This suggests that *N* promoter induction occurs either prior

Table 2. The effect of low temperature on the LUC activity in tobacco SLUCP 6-1 plants

Time	24°C (RLU), average	4°C (RLU), average	Change as compared with 24°C, %
0 h	18.68	17.38	−7.48
15 min	19.08	22.40	+17.37
30 min	19.23	22.93	+19.21 ($p < 0.05$)
1 h	20.99	24.75	+17.94
1 h 30 min	16.53	20.63	+24.81
2 h	18.60	14.99	−19.42
2 h 30 min	18.73	19.44	+3.79
3 h	18.01	18.58	+3.16
3 h 30 min	19.83	16.95	−14.52
4 h	18.25	20.75	+13.69
4 h 30 min	17.29	17.92	+3.63
5 h	19.35	16.66	−13.89
5 h 30 min	20.38	12.36	−39.36
6 h	19.46	15.66	−19.51
6 h 30 min	19.60	10.45	−46.71 ($p = 0.019$)
7 h	16.40	12.46	−24.04

Tissue was collected from the tobacco N::LUC plants treated at 24°C and 4°C. The averages of RLU were calculated from 4 plants per time point per treatment, done in triplicates with two technical repeats.

Table 3. The effect of TMV on the LUC activity in tobacco SLUC P6-1 plants

Time	Buffer (RLU), average	Virus (RLU), average	Change as compared with buffer, %
0 h	39.58	41.36	+4.49
15 min	42.50	39.84	−6.26
30 min	41.25	48.88	+18.48
1 h	36.94	53.93	+45.98 ($p < 0.05$)
1 h 30 min	39.15	53.41	+36.42 ($p < 0.05$)
2 h	41.66	49.19	+18.07
2 h 30 min	35.13	52.20	+48.61 ($p < 0.05$)
3 h	39.73	41.44	+4.30
3 h 30 min	34.05	37.56	+10.32
4 h	36.72	40.40	+10.04
4 h 30 min	40.08	40.42	+0.84
5 h	33.73	44.58	+32.16
5 h 30 min	40.88	44.24	+8.21
6 h	36.20	48.06	+32.77
6 h 30 min	41.26	49.93	+21.02 ($p < 0.05$)
7 h	48.74	44.17	+9.38

N::LUC tobacco plants were analyzed for the effect of virus and control on the specific activity of luciferase. The table indicated the averages from 4 treated plants (done in triplicates with two technical repeats) at various time points and the difference in luciferase activity of virus and control plants.

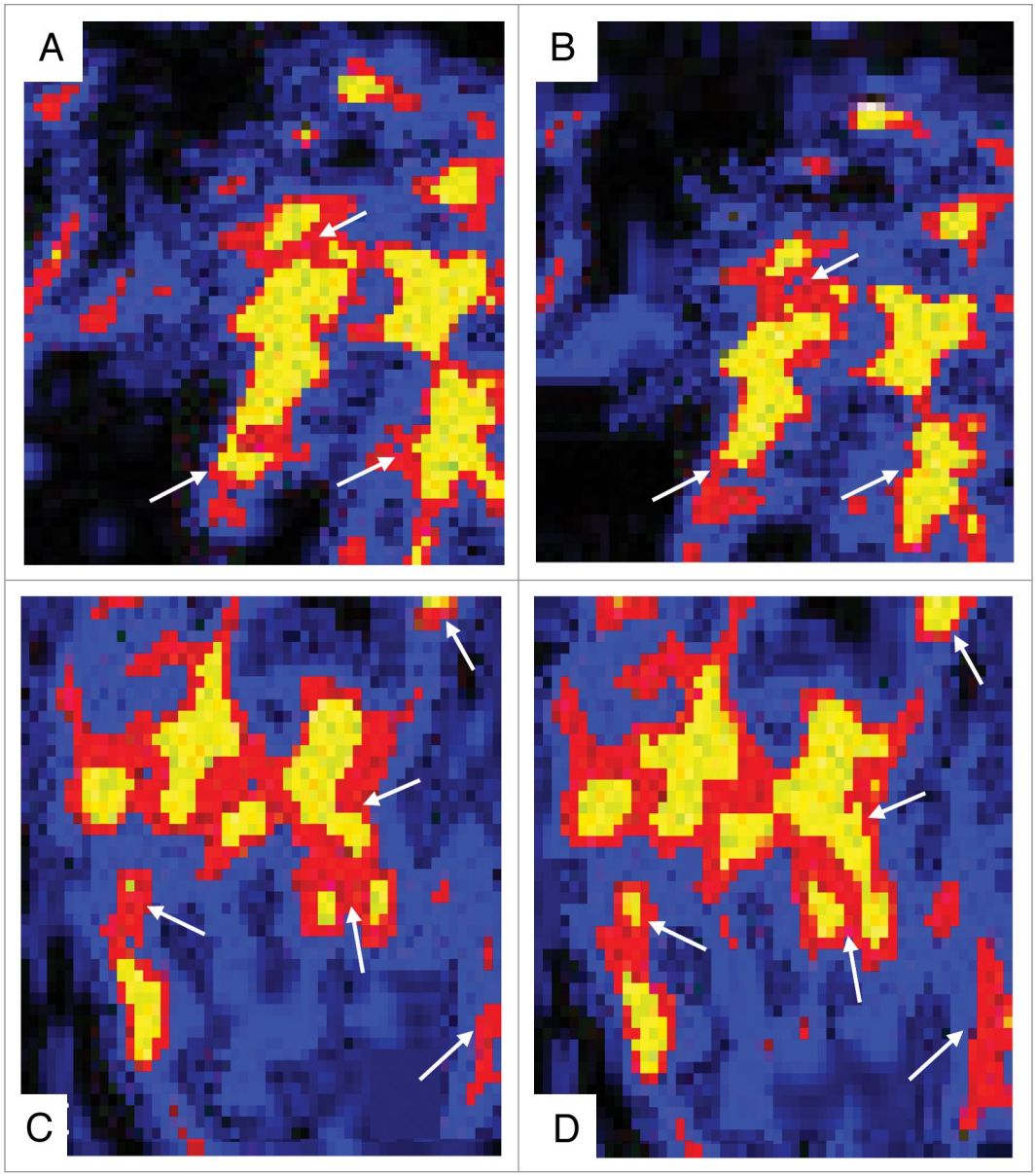

Figure 4. Luciferase expression and activity in virus infected and high temperature exposed plants. (**A**) Relative luciferase expression determined by qRT-PCR. Data points represent an average of the expression levels in two different plants and two technical replicates per treatment determined at indicated time points and error bars show the error progression of the standard deviations from the technical replicates. (*Student's t-test $p < 0.05$; **Student's t-test $p < 0.01$). (**B**) One tobacco SLUC P6-1 plant from virus and buffer treatment and two plants from control treatment were analyzed simultaneously. Virus (upper left), buffer (upper right) and control plants (bottom) incubated for 2 h at 24°C.

to induction of SA production or is part of a different response pathway altogether.

Very little information is available about the structure and the regulation of the N gene promoter. Previous study has indicated the existence of two cis-regulatory elements regulated by p50 alone (–269 to –250), or a combination of p50 and N protein pair (–290 to –271).[23] We have analyzed the 2,000 nt region (–1 to –2,000) of the *N* gene promoter and identified several potential cis-regulatory elements that are potential targets of various transcription factors responsive to stress (**Fig. S5** and **Table S7**). Among those were two heat shock elements (HSE; GAAAATTCT/AGAATTTTC) at the position of –165 to –157 ng, as well as a dehydration-responsive elements (DRE; TACCGACAT), CBF regulatory elements (cold regulated), ABA-responsive elements (ABRE; ACGTGG/TC), low temperature responsible element (LTRE), C-repeat/dehydration-responsive element binding factor (CBF) and Dc3 promoter-binding factor elements (DPBF). HSE, CRT/DRE/CBF, LTRE and DPBF elements were of particular importance as they are located within the first 1,000 nt of the promoter region (–1 to –1,000). As these cis-elements are regulated by environmental stress, they may be of critical importance for the regulation of *N* gene by TMV infection or/and temperature changes. It remains to be shown which of these elements are of any importance.

Materials and Methods

Plant growth conditions. Seeds of tobacco plants (*Nicotiana tabacum*) were grown in potting soil in a growth chamber (Enconair ecological chambers Inc.) at 24°C under 16:8 h of light:dark regime, with 80% humidity and light intensity of 200 $\mu Mm^{-2}s^{-1}$.

Plant transformation. *Vector description.* The transformation vectors were developed from the pCAMBIA1300 binary vector system carrying a kanamycin selectable marker for bacteria and hygromycin marker for plants. For the N::LUC and N::GUS fusions, the 4.1 kb cassette carrying the *N* gene promoter was cloned at 5'end of the promoterless LUC or GUS gene. This DNA fragment of 4.1 kb in size, containing *N* gene promoter was the same that have been used in previous studies. The total size of the pCAMBIA N::LUC vector was 14.4 kb and the total size of the pCAMBIA N::GUS vector was 15.3 kb (see **Fig. S2**).

Modified leaf-disc transformation. Agrobacterium tumefaciens (strain GV3101) was transfected with the binary vectors, pCAMBIA-N::GUS and pCAMBIA-N::LUC via electroporation. Resulting single colonies were used to prepare an overnight liquid culture in yeast extract-peptone (YEP) medium plus antibiotics (rifampicin 25 mg/L, gentamycin 25 mg/L and kanamycin 50 mg/L).

Tobacco plants (*Nicotiana tabacum,* cv Petite Havana SR1) were transformed by a modified leaf-disc transformation method described previously.[26] Briefly, an overnight culture of *A. tumefaciens* was harvested and resuspended in 10 mM $MgSO_4$, 5% sucrose and 0.005% Silwett L-77. Leaves were injured by inducing cuts and were immersed in *A. tumefaciens* suspension for 10 min. These leaves were co-cultivated for 48 h on MS medium,[27] and grown on plates until visible bacterial growth was formed around the leaf edge. After 48 h, the leaves were washed with sterile water and transferred to callus inducing medium (0.8 mg/L indole acetic acid, 2.0 mg/L kinetin in MS media supplemented with 30 mg/L hygromycin and 200 mg/L carbenicillin). The leaves were incubated on this media for 3–4 wk, during which the *A. tumefaciens* transformed cells produced calli and preliminary shoot formation took place. Each individual plantlet was separated and transferred to root inducing media (0.5 mg/L naphthalic acetic acid in MS media supplemented with 30 mg/L hygromycin and 200 mg/L carbenicillin). After 1–2 wk, the roots were induced from the plantlets and they were transferred to soil for subsequent growth.

Plant screening. *Screening for herbicide-resistance plants.* Seeds were sterilized and sown on MS medium containing 30 mg/L hygromycin. Resistant T0 seedlings were transferred to soil 3 wk post-germination. At T1 generation, plants were screened for segregation and plants showing 3:1 segregation were selected as containing a single locus insertion. These plants were analyzed for homozygosity by testing the hygromycin resistance in the progeny. Homozygous T2 plants were propagated and used for further experiments.

Screening for reporter gene expression. All herbicide resistant plants were tested for the expression of the reporter genes. For N::LUC lines, a charges couple device (CCD) camera (Gloor Instruments) was used (**Fig. S3A**), while for the N::GUS lines, a histochemical staining method was used (**Fig. S3B**). Plants showing uniform and consistent expression throughout the T0, T1 and T2 generations were used for further experiments.

Table 4. The effect of TMV and high temperature on the LUC activity in tobacco SLUC P6-1 plants

Time	Buffer (RLU), average	Virus (RLU), average	Change as compared with buffer, %
0 h	14.33	13.84	−3.40
15 min	15.38	14.45	−6.43
30 min	16.19	19.72	+21.80
1 h	19.22	19.96	+3.88
1 h 30 min	16.89	23.32	+38.07 ($p < 0.05$)
2 h	18.28	26.83	+46.78 ($p < 0.05$)
2 h 30 min	22.39	28.28	+26.32
3 h	23.42	20.42	−12.81
3 h 30 min	17.53	19.69	+12.35
4 h	20.48	21.89	+6.90
4 h 30 min	21.43	18.20	−15.07
5 h	25.59	22.18	−13.36
5 h 30 min	22.93	24.36	+6.26
6 h	20.84	21.20	+1.74
6 h 30 min	17.13	15.60	−8.95
7 h	19.87	16.50	+16.93

The tobacco N::LUC plants were treated with TMV or buffer, at 32°C. These plants were incubated at 24°C and the activity of the luciferase was analyzed. The table shows the averages of the expression of the plants in each treatment at various time points and the difference in comparison to control, done in triplicates with two technical repeats.

Analysis of the expression and activity of the GUS and LUC reporter genes. *GUS expression analysis using histochemical staining.* The procedure used for the histochemical analysis was published previously.[26] The staining buffer consisted of 100 mg 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc; Jersey Labs Inc.) with 0.05% NaN_3, 1 mL dimethylformamide and 0.05% Tween 80 in 200 mL of 0.1 M sodium phosphate buffer (pH 7.0). Tobacco plants were placed in tubes (1.5–15 ml), suspended in the staining buffer and vacuum infiltrated for 15 min. Subsequently, plants were placed at 37°C for 48 h. This was followed by destaining with 70% ethanol at 37°C. Ethanol was changed frequently until the plants were sufficiently bleached.

Luciferase expression analysis using quantitative real-time PCR. Line SLUC P6-1 was used for these experiments. For luciferase expression analysis using qRT-PCR, tissues were collected at indicated time points and flash frozen in liquid nitrogen. RNA was isolated using Trizol® Reagent (Invitrogen). In the experiments including TMV infection and or mock-treatment, only treated tissues were taken for the analysis. To prevent contamination with genomic DNA, RNA samples were cleaned up using the illustra RNAspin Mini kit (GE Healthcare). First strand cDNA synthesis was performed using the iScript™ Select cDNA Synthesis Kit (BioRad).

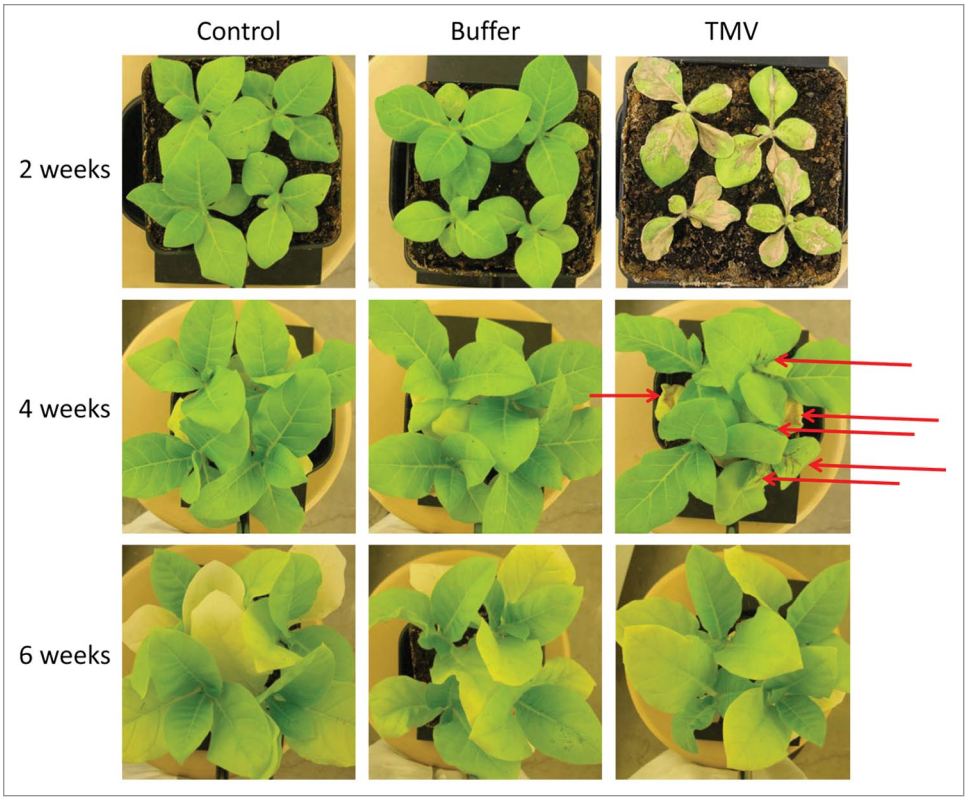

Figure 5. Response of tobacco plants to TMV at 2-, 4- and 6-wk-old plants. Images of control, buffer-treated and infected plants, treated at 2-, 4- and 6 wk of age. Images were taken 2 wk after treatment. Red arrows show necrotic lesions in the infected leaves.

qRT-PCR was performed using SsoFast™ EvaGreen® Supermix (BioRad) and Primers specific for the luciferase gene sequence: Luc fwd (5'-CTA TGT CTC CAG AAT GTA-3') and Luc rev (5'-AGG TCC TAT GAT TAT GTC-3') as well as several reference genes: Actin (fwd: 5'-TTG TGT TGG ACT CTG GTG ATG GTG-3'; rev 5'-AAT GGT GAT CAC CTG CCC ATC TGG-3'), L25 ribosomal protein (fwd: 5'-CCC CTC ACC ACA GAG TCT GC-3'; rev: 5'-AAG GGT GTT GTT GTC CTC AAT CTT-3') and EF1α (fwd: 5'-TGA GAT GCA CCA CGA AGC TC-3'; rev: 5'-CCA ACA TTG TCA CCA GGA AGT G-3'). Both L25 and EF1α primer sequences were described before.[28]

qRT-PCRs were performed using a CFX96™ Real-Time System on a C1000™ Thermal Cycler (BioRad). Analysis and normalization was done using the qbase^{PLUS} software.[29] All experiments included biological triplicate and technical duplicate and statistical significance was determined by pairwise Student's t-test.

Luciferase activity analysis using CCD camera. CCD camera-based luminescence detection was used for the spatial analysis of luciferase activity. For this plants were sprayed with a luciferin solution, which contained 1 mg of luciferin powder dissolved in dimethyl sulfoxide and adjusted to a final concentration of 0.5 mM with 0.25 mM sodium phosphate and 0.1% Tween20. Plants were incubated with Luciferin solution for 30 min in dark. They were then placed in the CCD camera chamber and images were taken using the Pixcel software 2.8 (PerkinElmer). One control image with light was taken first. Two subsequent dark images were taken with 10 min exposure each. These images were processed using the analySIS software 3.2 (Soft Imaging Systems). In brief, the control image taken in the presence of light was given green color (**Fig. S4A**). The two dark images were superimposed to remove background luminescence. A color code was defined to give gradual intervals of the intensity of LUC activity (**Fig. S4B**). This was required to analyze any minute change in the intensity of the LUC activity over time.

Specific luciferase activity analysis using a luciferase quantification assay. The luciferase quantification assay was performed as described before,[30] with modifications. For analysis, plant tissue was collected and flash frozen in liquid nitrogen. The tissue was ground to a fine powder in liquid nitrogen and homogenized with 500 μL of LUC extraction buffer. The homogenate was centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant was taken to a fresh tube. Total protein concentration was measured for these samples using the Nano Orange Protein Quantification Kit (Invitrogen). Briefly, for each sample, 195 μL of Nano Orange working solution was added per well of a microplate and mixed with 5 μL of the extract. The plate was incubated at 90°C for 10 min and cooled down to room temperature. The amount of fluorescence was measured using a microplate reader (FL×800, Bio-Teck Instruments Inc.). Various concentrations of bovine serum albumin (BSA) were used for a standard curve. The sample extracts were standardized to contain the same total protein concentration by diluting them with LUC extraction buffer. In a microplate, 100 μL of LUC assay buffer and 50 μL of the sample extract were added per well. The plate was placed in the carrier of microplate reader and 50 μL of 1 mM of luciferin solution were added to each well to a final concentration of 0.25 mM. The luminescence reading was recorded immediately using the KC4 software 3.02 revision 4 (Bio-Teck Instruments Inc.). Luciferase activity was recorded as an endpoint reading in the luminescence mode. Data are represented in relative luminescence units calculated by the formula luminescence units per mg of total protein.

TMV inoculation of N::LUC plants. *TMV inoculation for subsequent analysis of LUC activity.* Lines SLUC P2-1 and SLUC P6-1 were used for the experiment. Plants were grown in growth chambers for 6–8 wk. TMV strain U1 in a concentration of 5 μg/mL (in 10 mM sodium phosphate buffer, pH 7.0) was used for infection. Two types of inoculation and analysis were performed.

In the first set of experiments, luciferin was applied to single leaves and leaves were incubated for 30 min in the dark. This leaf was then infected with TMV (50 ng per single infection site) or mock-treated with help of carborundum powder and the residues were washed with sterile water. The CCD camera was then fine focused on the leaf for very detailed pictures. Continuous dark frames were taken in the region every 2 min for 2 h post-infection. All the frames were processed as mentioned before and analyzed for any minute changes in LUC expression (see the **Supplementary Movie S1**).

In the second set of experiments, carborundum powder and 50 μL of distilled water were rubbed gently into the selected leaves. Residues were washed away using a spray of sterile distilled water. Twenty μL of the virus (100 ng) was then gently rubbed into the injured area. For a control treatment, 20 μL of buffer (10 mM sodium phosphate, pH 7.0) was used. Tissues were collected at indicated time points for further analysis.

The analysis of the effect of TMV on tobacco plants of different age was performed using 100 ng of TMV per each inoculated leaf (two leaves per plant). Infection was done on 2-, 4- and 6-wk-old plants at 32°C and pictures were taken 2 wk later.

Analysis of the effect of temperature extremes on LUC activity. SLUC P2-1 and SLUC P6-1 plants were grown under normal growth conditions in a growth chamber until 4–8 wk of age. They were divided into three treatments. For the high temperature treatment, plants were shifted to a chamber set to 45°C, whereas for the low temperature, plants were placed at 4°C. Control treatment plants were maintained under control conditions in the chamber at 24°C. At 0, 15 min, 30 min, 1 h, 1 h 30 min, 2 h, 3 h, 4 h and 6 h time points, reporter expression analysis was done from four plants from each treatment using the CCD camera. For quantification assay, tissues from the treated plants were collected at aforementioned time points and flash frozen. For RT-PCR analysis, tissues were collected at 0 min, 15 min, 30 min, 1 h, 3 h and 6 h time points and flash frozen.

Analysis of the effect of salicylic acid on the LUC activity. The SA solution consisted of 5 mM SA in distilled water with 0.1% Tween 80. As a control, distilled water containing 0.1% Tween 80 was used. The N::LUC plants were grown until 6–8 wk of age. For the SA and control treatments, SA and mock solution was uniformly applied on the leaves by using a fine spray. At 0, 15 min, 30 min, 1 h, 1 h 30 min, 2 h, 3 h, 4 h and 6 h time points, reporter expression analysis was done from four plants from each treatment using the CCD camera. For quantification assay, tissues from the treated plants were collected at aforementioned time points and flash frozen.

Analysis of cis-regulatory elements in the *N* gene promoter. To localize transcription factor binding sites (TFBSs) in the promoter region of the *N* gene, PLACE database (a database of plant cis-acting regulatory elements, www.dna.affrc.go.jp/PLACE/signalscan.html) was used.[31] The localizations of TFBS were also confirmed by scanning the promoter sequence using patmatch.pl Perl script (references below).[32] For promoter analysis, we used 2,000 nt sequence immediately upstream of the ATG.

Statistical treatment of the data. All experiments included biological triplicate and technical duplicate and statistical significance was determined by pairwise Student's t-test or one-way ANOVA. **Tables S2–6** show details of statistical analysis.

Table 5. The effect of salicylic acid on the LUC activity in tobacco SLUC P6-1 plants

Time	Buffer (RLU), average	SA (RLU), average	Change as compared with buffer
0 h	10.65	10.29	−3.40
15 min	10.11	10.66	+5.44
30 min	10.37	11.60	+11.91
1 h	12.05	10.61	−11.91
1 h 30 min	10.95	11.51	+5.07
2 h	11.24	11.96	+6.48
2 h 30 min	12.07	10.80	−11.76
3 h	12.37	12.08	−2.34
3 h 30 min	11.59	12.60	+8.76
4 h	11.38	13.00	+14.28
4 h 30 min	9.78	11.13	+13.83
5 h	12.10	12.35	+2.02
5 h 30 min	11.98	13.12	+9.52
6 h	12.29	12.41	+0.96
6 h 30 min	10.78	11.28	+4.66
7 h	12.05	11.18	−7.22

Tobacco N::LUC plants were treated with either salicylic acid or buffer. From four plants from each time point, tissues were sampled and the luciferase activity was measured. The table shows the activity at various time points after the treatment and the differences in the control and SA treatment, done in triplicates with two technical repeats.

Conclusion

The function of the *N* gene promoter region is developmentally regulated. The results suggest that the N protein might not be present in leaf lamella during early growth phases. Also, the roots may lack the N protein throughout the growth phase. In such a scenario N protein mediated resistance response might be absent from the lamella of young plants and the roots of plants of different ages. Experiments with inoculation of TMV in these tissues might reveal validity of this postulation. Infection with TMV leads to an immediate increase in the activity of LUC driven by *N* gene promoter. This induction may be important for development of resistance. The role of this induction and mechanism responsible for it still need to be revealed. High but not low temperatures can influence the function of *N* gene promoter, whereby these temperature extremes can hinder its induction. The results suggest that there is no influence of SA on *N* gene promoter region. Hence, it can be concluded that the efficiency of the *R* genes might be subjective to various biotic and abiotic factors. The recognition of these influences may aid in understanding host pathogen interactions and development of pathogen resistant plants.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary Material

Supplementary material may be found here:
https://www.landesbioscience.com/journals/psb/article/24760/

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