

Full Length Research Paper

## Resistance-related gene transcription and antioxidant enzyme activity in *Nicotiana* spp. resistant to anthracnose

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The two tobacco relatives of *Nicotiana alata* and *Nicotiana longiflora* display a high level of resistance against *Colletotrichum nicotianae* and the two genes *NTF6* and *NtPAL* related to pathogen defense transcription were higher in *N. alata* and *N. longiflora* than the commercial cv. K326. Inoculation with *C. nicotianae* reduced the abundance of *NTF6* and *NtPAL* transcript during the first 48 h post inoculation (hpi) when only biotrophic hyphae were present, but increased it over the following 24 h as necrotrophic hyphae began to predominate. Activity levels of phenylalanine ammonia lyase, peroxidase and polyphenol oxidase changed markedly at 72 hpi. The conclusion was that the pathogen enters the host leaf within 24 hpi, triggering the up-regulation of various defense-related genes in a resistant host plant. By 72 hpi, the pathogen switched to necrotrophic growth to avoid contact with the increasing presence of host defense compounds.

**Key words:** *Nicotiana alata*, *Nicotiana longiflora*, tobacco anthracnose resistance-related gene, antioxidant enzyme activity.

### INTRODUCTION

Plants have evolved a multifaceted system of defense against pathogens, some of which are highly specific to a particular microbial species (Doehlemann et al., 2008). The host response typically involves a concerted series of events ranging from the rapid generation of reactive oxygen and nitrogen species, through changes in ion flux across the plasma membrane, proteolysis, and the reprogramming of hundreds of genes (Dangl and Jones, 2001). Although, many components of the plant defense signal transduction pathway remain obscure, those responsible for both salicylic acid-dependent systemic acquired resistance and jasmonate- and ethylene-induced resistance are well documented (Hammond-Kosack and

Parker, 2003; Bari and Jones, 2009). For biotrophic pathogens, effectors trigger the plant's defense responses and salicylic acid signaling is used to initiate apoptosis at the site of the pathogen's entry. The dying cells can, however, support the growth of necrotrophic pathogens (Doehlemann et al., 2008). The defense response therefore has evolved such that the salicylic acid-dependent pathway is largely activated by biotrophic pathogens, while the jasmonate- and ethylene-dependent pathway operates against necrotrophic pathogens and insects (Qiu and Wang, 2007). Associated with the defense signal transduction network, plants have also evolved a set of antioxidation strategies which are brought into play to miti-

gate against the deleterious effects of reactive oxygen species. Prominent among the enzymes involved in reactive oxygen species scavenging are phenylalanine ammonia lyase (PAL), various peroxidases (POD) and various polyphenol oxidases (PPO) (Averyanov, 2009).

PAL features in the phenylpropanoid metabolic pathway, which is responsible for lignin synthesis (Galis et al., 2006; Ferrareze et al., 2013). POD enzymes may also participate in lignin and suberin synthesis, as well as acting to underpin the production of various molecules endowed with antibiotic properties (Goodman et al., 1986; Averyanov, 2009). Increased levels of POD activity have been observed in conjunction with both local and systemic disease resistance (Karban et al., 1989; Kombrink and Somssich, 1995). PPO catalyzes the oxygen dependent oxidation of phenolics to quinines (Li and Steffens, 2002). The extent of the damage caused by reactive oxygen species depends to a large extent on the level of coordination among the various scavenging enzymes (Hao et al., 2012). Tobacco anthracnose, the causative agent of which is the hemibiotrophic fungus *Colletotrichum nicotianae* (Lucas, 1965), is a highly destructive pathogen of tobacco seedlings. *C. nicotianae* is classified as a hemibiotroph because it initially establishes a biotrophic interaction with its host before eventually switching to its destructive necrotrophic lifestyle (Shen et al., 2001). Two close relatives of cultivated tobacco, namely *Nicotiana glauca* and *Nicotiana glauca*, which are grown mainly as ornamentals, have been shown to be extremely resistant to anthracnose. Here, we set out to elucidate the mechanisms controlling their resistance by comparing their transcription profiles of known defense-related genes, and the activity levels of their antioxidant enzymes.

A better understanding of the gene network underlying anthracnose resistance in these tobacco relatives may help elaborate resistance breeding strategies in the commercial crop species.

## MATERIALS AND METHODS

### Fungal inoculation

An isolate of *C. nicotianae* was cultured on potato dextrose agar (PDA; 39 g of PDA, 1 L of water) under continuous fluorescent light at 25°C for about seven days until the surface of the plate was almost covered with mycelia as well as conidia. Each Petri plate of culture was flooded with 10 ml sterilized distilled water and conidia were released by scraping with a metal spatula. The resulting suspension was filtered through two layers of cheesecloth and 0.02% (v/v) Tween 20 was added as a surfactant. The concentration of conidia was adjusted to  $\approx 1 \times 10^7$  spores/ml by hemacytometer prior to inoculation. Tobacco seedlings were grown at 24°C and a relative humidity of 30% under a 16 h photoperiod with a photosynthetically active radiation intensity of 200  $\mu\text{mol s}^{-1}\text{m}^{-2}$ . At the six leaf stage, leaves were rinsed in sterile water and coated with the *C. nicotianae* conidia suspension. Negative control leaves were rinsed in 0.02% Tween 20. After inoculation, the seedlings were enclosed in a plastic bag and held at 26°C for 24 h in the dark, and then immediately transferred into a 24°C 14 h photoperiod, 95% relative humidity regime with the bag removed. Leaf samples were harvested immediately and then after a set number of hours (as below) depend-

ing on whether the assay was for gene transcript or gene product.

### Resistance to anthracnose diseases

We evaluated resistance of the two tobacco relatives of *N. glauca*, *N. longiflora* and cv. K326 to anthracnose. Susceptibility to anthracnose infection was evaluated at 30 days after inoculation based on Chen et al., (1997) disease index. In order to explore the resistance of two tobacco *N. glauca* and *N. longiflora* to the anthracnose pathogen. Using the percent disease index as the reference standard, the percent disease index was scored after inoculation in intensity scale of 0 to 5: No infection = 0, 0.1 to 11%; leaf area affected = 1, 11.1, -33%; leaf area affected = 2, 33.1 to 55%; leaf area affected = 3, 55.1 to 77%; leaf area affected = 4, 77.1%; leaf area affected = 5. Percent disease incidence (PDI) was calculated based on the following formula:

$$PDI = \frac{\text{Sum of all numerical grades}}{\text{Total number of leaves counted} \times \text{Maximum grade}} \times 100$$

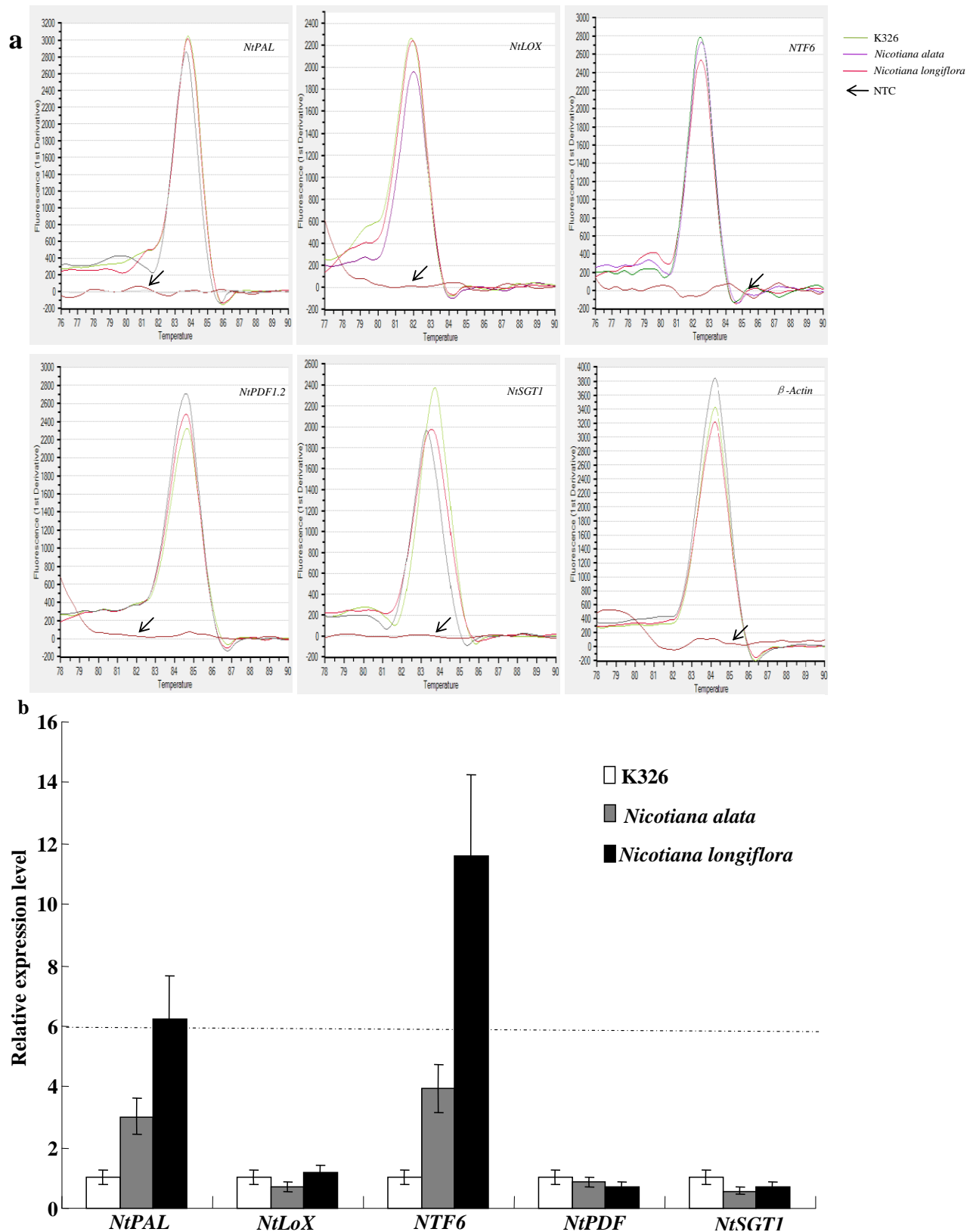
### Primer design, RNA isolation and reverse transcription

Primer pairs of genes (Table 2) were designed using Primer Premier 5.0 software under default parameters and were custom-ordered from a commercial supplier (Invitrogen, Guangzhou). The specificity of each primer was confirmed by melting-curve analysis after amplification for the six genes (Figure 1a). Standard curves using a dilution series of the cDNA (from each species of tobacco and spanning six orders of magnitude) were made to calculate the gene-specific PCR efficiency and regression coefficient ( $R^2$ ) for each gene (Table 2). Total RNA was extracted from leaf tissue at each time point (0, 1, 2, 3, 6, 12, 24, 48 and 72 hpi) using the TRIzol reagent (Invitrogen) following the manufacturer's protocol. This preparation was treated with RQ1 RNase-Free DNase (Promega) before conversion to cDNA using a M-MLV reverse transcriptase kit (Promega). Two independent reverse transcription (RT) reactions were pooled from each leaf processed (three biological replicates per harvest time). The subsequent quantitative real time (qRT) PCRs were run as three technical replicates per each pooled cDNA. The qRT PCRs were implemented on a TP900 Thermal Cycler Dice™ Real Time System (TakaRa) using a SYBR Premix Ex Taq kit (TaKaRa). Specific primers were developed for five separate defense-related genes based on public domain sequences (Table 2). Each PCR comprised 12.5  $\mu\text{L}$  SYBR® Green Real-time PCR Master Mix (TaKaRa), 1.0  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ), 9.5  $\mu\text{L}$  ddH<sub>2</sub>O and 1  $\mu\text{L}$  cDNA. The reactions were subjected to 40 cycles of 95°C/30 s, 58°C/30 s, 72°C/60 s.

The fluorescence signal detected at the end of each extension step was used to generate an amplification profile. The transcription data were normalized against those obtained by monitoring tobacco  $\beta$ -actin using the  $\Delta\Delta\text{CT}$  method (Livak and Schmittgen, 2001).

### PAL, PPO and POD extraction and activity measurement

Leaves were harvested at give the sampling hpi's, frozen in liquid nitrogen, and ground to a fine powder, which was stored at -80°C until required. The assay for PAL was derived by extracting 0.25 g leaf powder with 1 ml 0.1 M sodium borate buffer (pH 8.8) for 30 min at 4°C, while those for both PPO and POD were obtained from 0.20 g powder extracted in 1 ml 0.1 M sodium phosphate buffer (pH 6.8) for 30 min at 4°C. The suspensions were centrifuged (10,000 g, 15 min, 4°C) and the crude supernatant used to assay for the activity of PAL, PPO and POD. PAL activity was determined by mixing 3.4 ml deionized water, 0.6 ml of a 100 mM solution of L-phenylalanine, and 2 ml of the solution containing the PAL extract. The mixture was kept in a water bath at 40°C for 45 min and it was read at 290 nm in a spectrophotometer (Shimadzu UV-2550) against a blank made with



**Figure 1.** Specificity of primer pairs for RT-qPCR amplification. Melt curve showed a single peak for each selected reference gene and no amplicon was observed in no template control (NTC) indicated by arrows (a), real-time qRT-PCR analysis of the transcription of selected defense-related genes in three *Nicotiana* spp. The  $\beta$ -actin gene was used as a reference. Data expressed as transcription abundances relative to that present in cv. K326, and are given in the form mean  $\pm$  standard error (SD). Dashed lines indicate the 6-fold threshold level (b).

4 ml water and 2 ml of the solution containing the PAL extract. Enzymatic activity is reported as micromoles of cinnamic acid/milligrams of protein (Gonzalez-Aguilar et al., 2004). PPO activity was determined using a spectrophotometric method based on an initial rate of increase in absorbance at 410 nm. Phosphate buffer solution pH 7 (0.1 M, 1.95 ml), 1 ml of 0.1 M catechol as a substrate and 50  $\mu$ L of the enzyme extract were pipetted into a test tube and mixed thoroughly. Then, the mixture was rapidly transferred to a 1-cm path length cuvette. The absorbance at 410 nm was recorded continuously at 25°C for 5 min using ultraviolet-visible spectrophotometer, Shimadzu UV-2550 (Flurkey, 1985). POD activity was assayed spectrophotometrically at 470 nm (Shimadzu UV-2550) using guaiacol as a phenolic substrate with hydrogen peroxide.

0.1 ml of leaf extract was mixed with 2.9 ml of 25 mM sodium acetate buffer, pH 4.5, and 0.05 ml of 20 mM guaiacol. The reaction was started by addition of 0.01 ml of 40 mM  $H_2O_2$ , and the initial rate of increase of absorbance at 470 nm was measured. One milli-unit of enzyme is the amount causing an absorbance increase of 1.0 per min at 24°C (Rathmell and Sequeira, 1974).

### Statistical analysis

Means were compared using the Student's *t* test implemented in SPSS v11.5 for Windows (Microsoft). Data are given in the form of mean  $\pm$  standard error (SD).

## RESULTS

### The resistance to anthracnose diseases

The results show that the disease index of cv. k326 was 74.17%, and the two tobacco relatives of *N. alata* and *N. longiflora* were 23.33 and 26.67%, which displayed high levels of resistance against the anthracnose pathogen (Table 1).

### The transcription of defense-related genes

The transcription of all five target genes (*NtPAL*, *NtLox*, *NTF6*, *NtPDF* and *NtSGT1*) was detectable in mock-inoculated *N. alata*, *N. longiflora* and cv. K326. The abundance of both *NtPAL* and *NTF6* transcript was higher in *N. alata* and *N. longiflora* than in cv. K326, whereas that of the other three genes was comparable across the three hosts (Figure 1b). *NtPAL* and *NTF6* were therefore targeted for a temporal analysis of transcription following inoculation with *C. nicotianae*. In *N. alata*, *NTF6* transcription was induced by about 6.9 fold by 1 hpi; it decreased over the period 2 to 12 hpi, peaked again to 13.3 fold of background around 24 hpi, fell to its minimum level at 48 hpi and finally recovered to 0.8 fold of background by 72 hpi. In *N. longiflora*, the same gene was induced by 6.6 fold at 3 hpi, fell to 1.7 fold by 6 hpi and peaked to 81.2 fold of background at 12 hpi. In cv. K326, *NTF6* transcription was also induced by inoculation, peaking at 12 hpi but to a level substantially below that reached in either *N. alata* or *N. longiflora* (Figure 2a). The transcription response of *NtPAL* in *N. alata* and *N. longiflora* was also induced by the infection of pathogen (Figure 2b). The abundance of *NtPAL* transcript in *N. alata* was 21.0 fold above background at 6 hpi, less than background in the

period 12 to 48 hpi, but had recovered by 72 hpi. In *N. longiflora*, transcription peaked at 3.1 fold of background at 12 hpi, fell to a minimum level at 48 hpi, and had recovered partially by 72 hpi. The level of *NtPAL* transcription in cv. K326 was substantially lower than in either *N. alata* or *N. longiflora*, but peaked to 2.2 fold of background at 6 hpi.

### Antioxidant enzyme activity

PAL activity in *N. alata* reduced over the period 24 to 72 hpi, but then increased rapidly up to the activity peak of PAL was 16.18  $U \cdot g^{-1} Fw \cdot min^{-1}$  in the 168 dpi, higher 0.6 fold the level detected in the inoculated cv. K326. At all time points (except for 72 hpi, where the difference was significant), the activity level in the inoculated plants exceed that in the inoculated cv. K326 (Figure 3a). Both PPO and POD activity rose over the first 72 hpi, peaking at, respectively, 2.1 and 2.6 fold the level detected in the inoculated cv. K326 (Figure 3b and 3c). PAL activity in *N. longiflora* behaved in a similar fashion. Its level reached a minimum at 120 hpi, and was lower than in the inoculated cv. K326 by the end of the measurement period. The activity peak of PAL was 18.18  $U \cdot g^{-1} Fw \cdot min^{-1}$  in the 168 dpi, higher 0.8 fold the level detected in the inoculated cv. K326 (Figure 3a). PPO activity had fallen by 4.1 fold at 72 hpi and remained below that of the inoculated cv. K326 until 168 hpi (Figure 3b). Meanwhile, POD activity increased sharply over the first 24 hpi and then declined up to 72 hpi, thereafter recovering somewhat (Figure 3c).

## DISCUSSION

The MAP kinase encoding gene *NTF6* is a key regulator of SAR, since its silencing has been recorded as attenuating both the resistance of tobacco to tobacco mosaic virus and of tomato to *Pseudomonas syringae* (Liu et al., 2004; Ekengren et al., 2003). Here, *NTF6* transcription was found to be induced by infection with *C. nicotianae*, which suggests that similar, if not the identical MAP kinases are involved in the host's response to virus, bacterial and fungal pathogens, as proposed by Liu et al. (2004) and Bartels et al. (2009). The double peak of *NTF6* induction observed during the *C. nicotianae* infection process was remarkable. The first coincided with the fungal penetration stage (1 to 3 hpi) and a second, much stronger one with the formation of an infection vesicle in the host cell (12 to 24 hpi) (Shen et al. 2001). In contrast, in the susceptible host cv. K326, there was little or no evidence of induction at either of these time points. The inference is that resistant types such as *N. alata* and *N. longiflora*, but not susceptible ones such as cv. K326, are able to sense the occurrence of developmental changes in the pathogen and use this information to mount a successful defense. Such a qualitative difference between resistance and susceptibility is consistent with the consensus view that in a susceptible host, the defense response tends to be delayed and not very intense (Métraux et al.,

**Table 1.** Resistance of different cultivars tobacco to anthracnose.

Cultivar	Number of treatment	Disease index						Percent disease incidence (%)	Significant difference
		0	1	2	3	4	5		
<i>N. longiflora</i>	30	12	8	7	2	1	0	26.67	b
<i>N. alata</i>		13	9	6	1	1	0	23.33	c
K326		1	2	5	11	11	0	74.17	a

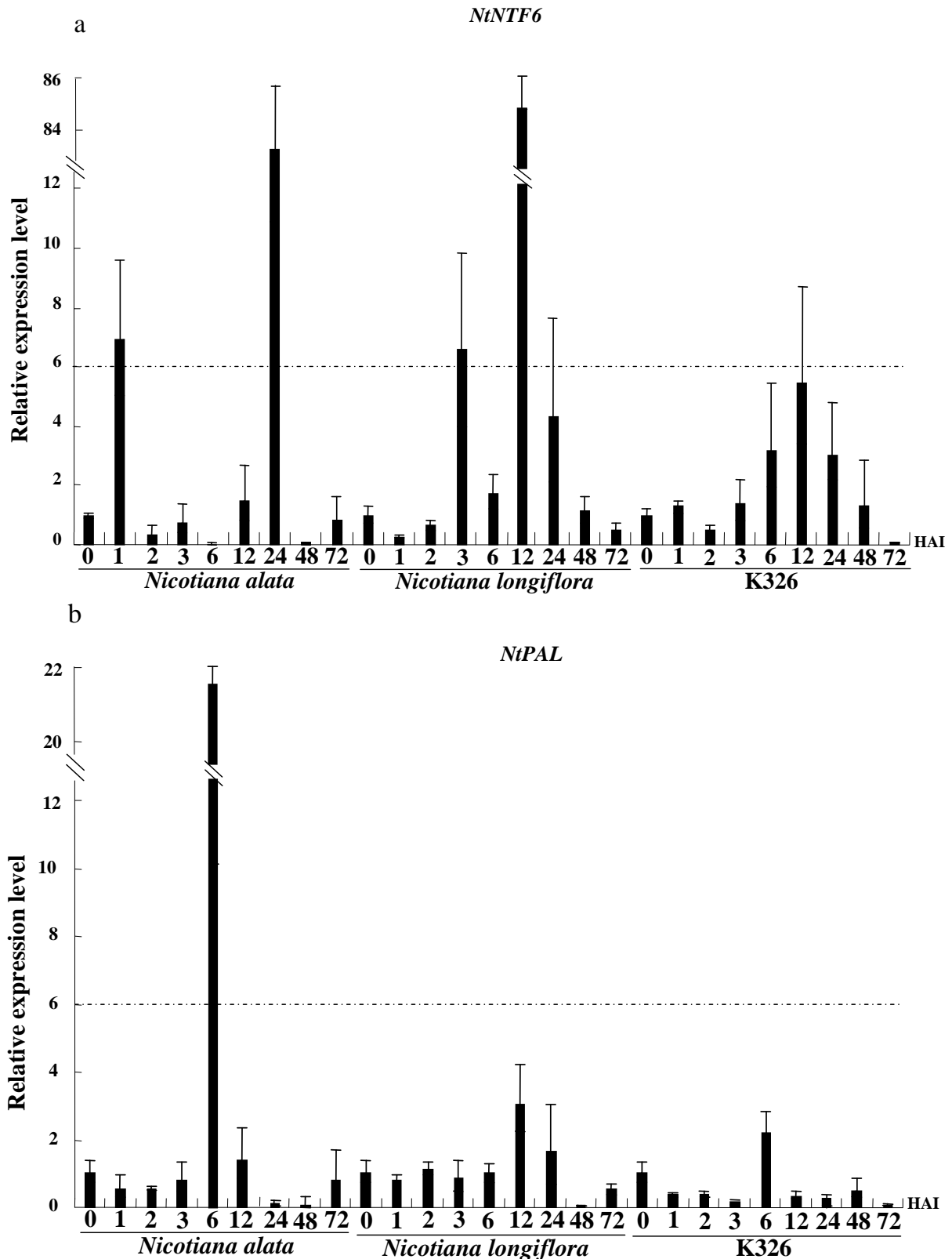
**Table 2.** Primers sequences and amplicon characteristics of RT-qPCR.

Gene name	Primer pair	GenBank	<i>Nicotiana alata</i>		<i>Nicotiana longiflora</i>		K326	
			Regression coefficient (R <sup>2</sup> )	Amplification efficiency (%)	Regression coefficient (R <sup>2</sup> )	Amplification efficiency (%)	Regression coefficient (R <sup>2</sup> )	Amplification efficiency (%)
<i>NtPAL</i>	5'-TCGGGCTTTCCATTCATCACC-3' 5'-AAGAAGCGTTCCTGTTGCTG-3'	AB289452	0.993	109.4	0.994	112.3	0.998	113.5
<i>NtLOX</i>	5'-GCCTATGCAGCAGTGAATGA-3' 5'-TGCCTATTTGTTGCGATCA-3'	X84040.1	0.995	103.5	0.997	105.2	0.999	102.1
<i>NtF6</i>	5'-GCTATTAGTTTCGCCAGAG-3' 5'-CCTCAACAGTTATGCGTTTATG-3'	AY547494.1	0.998	107.8	0.991	101.9	0.994	103.9
<i>NtPDF1.2</i>	5'-GGAAATGGCAAACCTCCATGCG-3' 5'-ATCCTTCGGTCAGACAAACG-3'	X99403.1	0.999	99.3	0.996	99.8	0.998	99.5
<i>NtSGT1</i>	5'-TCCCACTCGCCATCCTGAA-3' 5'-GTGCCGCAAATTCAACTCTG-3'	AF516180	0.994	105.7	0.995	103.6	0.997	105.9
<i>β-actin</i>	5'-ATGCCTATGTGGGTGACGAAG-3' 5'-TCTGTTGGCCTTAGGGTTGAG-3'	U60495	0.998	104.7	0.996	105.7	0.994	105.9

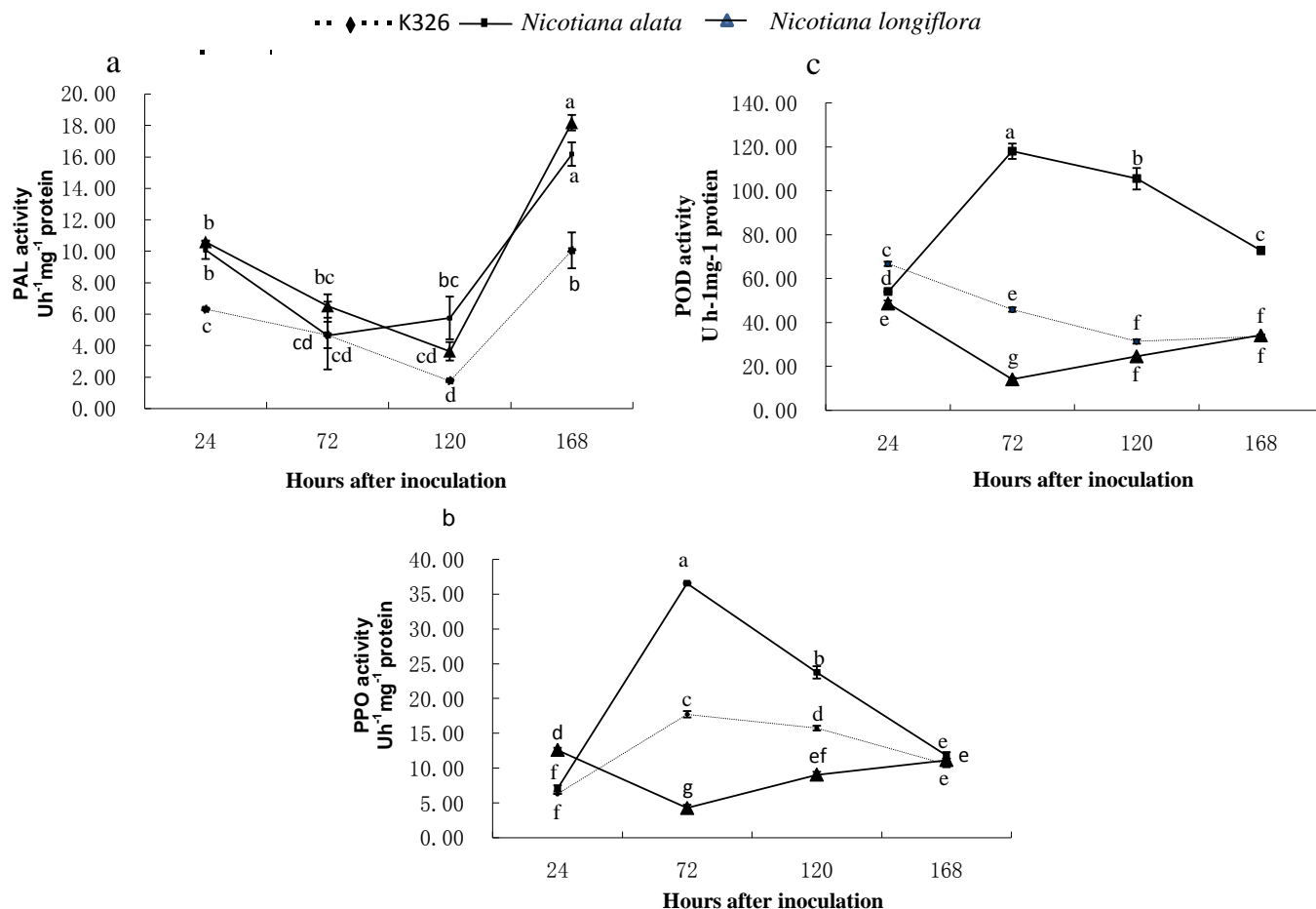
2009). *NtPAL* encodes the first committed enzyme in the phenylpropanoid pathway, which is responsible for the synthesis of both phytoalexins and lignin. Both these compounds are implicated in preventing the pathogen from penetrating the cell wall (Shadle et al., 2003; Sun et al., 2013). Here, *NtPAL* transcription was substantially induced at

an early stage in the infection process in *N. alata*, but was not induced in either *N. longiflora* or cv. K326. The differential response between the two resistant hosts implies that there is more than one specific mechanism underpinning resistance against *C. nicotianae* even within the same genus. Biotrophs such as *Uromyces vigneae* and *Uromyces maydis*,

as well as hemibiotrophs such as *Mycosphaerella graminicola* during their biotrophic phase, need to suppress or at least attenuate the host's defense response if they are to successfully parasitize the host (Panstruga, 2003; Caldo et al., 2006; Jones and Dangl, 2006). In maize infected with *U. maydis*, the initial response involves the activation



**Figure 2.** Transcription of (a) *NTF6* and (b) *NtPAL* following infection by *C. nicotianae*. Transcript abundance was quantified using real time qRT-PCR at a number of time points after inoculation. Transcript abundances were normalized with respect to that of the reference gene  $\beta$ -actin, and expressed relative to the level present in mock-inoculated plants for each time point. Values given in the form mean  $\pm$  SD. Dashed lines indicate the sixfold threshold level.



**Figure 3.** Defense response-related enzyme activity in *N. alata* and *N. longiflora* infected by *C. nicotianae*. **(a)** PAL, **(b)** PPO and **(c)** POD. Values given in the form mean  $\pm$  SD based on  $n = 3$  (two technical replicates). Different lower case letters at each point indicate statistically significant differences ( $P < 0.05$ ).

of a number of defense-related genes, but the effect is only transient, thereby allowing the establishment of the pathogen's biotrophic growth (Doehlemann et al., 2008). Here, we have shown similarly that in the tobacco *C. nicotianae* interaction, there was evidence for the transient activation of defense-related genes. Once the biotrophic growth of the pathogen became established (between 48 and 72 hpi), the transcription of both *NTF6* and *NtPAL* was substantially suppressed, and after this time point, necrotrophic hyphae began to predominate; thus, the developmental change in the pathogen was readily recognized by the host. The 72 hpi time point featured too in the antioxidant enzyme response. The probability is that during the first 24 hpi, the pathogen locates the stomatal cavities on the tobacco leaf and penetrates the leaf via the stomata. In a resistant host, this event stimulates the induction of a whole spectrum of defense-related genes. By 72 hpi, the pathogen has switched from biotrophic to necrotrophic growth, and thereby succeeds in avoiding exposure to an increasing concentration of defense compounds.

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