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Enhanced Resistance to Viruses in *Nicotiana edwardsonii* 'Columbia' Is Dependent on Salicylic Acid, Correlates with High Glutathione Levels, and Extends to Plant-Pathogenic Bacteria and Abiotic Stress

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Our earlier research showed that an interspecific tobacco hybrid (*Nicotiana edwardsonii* 'Columbia' [NEC]) displays elevated levels of salicylic acid (SA) and enhanced resistance to localized necrotic symptoms (hypersensitive response [HR]) caused by tobacco mosaic virus (TMV) and tobacco necrosis virus (TNV), as compared with another interspecific hybrid (*Nicotiana edwardsonii* [NE]) derived from the same parents. In the present study, we investigated whether symptomatic resistance in NEC is indeed associated with the inhibition of TMV and TNV and whether SA plays a role in this process. We demonstrated that enhanced viral resistance in NEC is manifested as both milder local necrotic (HR) symptoms and reduced levels of TMV and TNV. The presence of an adequate amount of SA contributes to the enhanced defense response of NEC to TMV and TNV, as the absence of SA resulted in seriously impaired viral resistance. Elevated levels of subcellular tripeptide glutathione (GSH) in NEC plants in response to viral infection suggest that in addition to SA, GSH may also contribute to the elevated viral resistance of NEC. Furthermore, we found that NEC displays an enhanced resistance not only to viral pathogens but also to bacterial infections and abiotic oxidative stress induced by paraquat treatments.

Keywords: glutathione, paraquat, pathogen resistance, salicylic acid-mediated resistance, tobacco, tobacco mosaic virus, tobacco necrosis virus

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Early pathogen recognition and the rapid reprogramming of the plant transcriptome during pathogenic attack are necessary to successfully prevent pathogen spread in plants by activating an array of defense responses. These processes may culminate in a resistance reaction that ultimately limits pathogen spread and is often associated with a localized programmed cell death (PCD) at infection sites (hypersensitive response [HR]) (Balint-Kurti 2019; Künstler et al. 2016). However, pathogen resistance can be also symptomless, as exemplified by extreme resistance, a rapid, efficient response of plant hosts limiting viruses (Bendahmane et al. 1999; Künstler et al. 2016; Sekine et al. 2008). In fact, it has been shown in several viral, bacterial, and fungal infections that plant resistance during an HR is often independent of cell or tissue death (Balint-Kurti 2019; Bendahmane et al. 1999; Cole et al. 2001; Künstler et al. 2016). Previously, we demonstrated that during an HR elicited by cauliflower mosaic virus (CaMV) in an interspecific tobacco hybrid (*Nicotiana edwardsonii*), resistance is governed by the product of a resistance (*R*) gene derived from one of the parents (*Nicotiana glutinosa*), whereas cell death is conditioned by the product of the gene *ccd1* (*CaMV cell death 1*) derived from the other parent (*Nicotiana clevelandii*), implying that in this particular plant–virus interaction, the resistance and cell death responses that comprise an HR can be genetically uncoupled (Cole et al. 2001; Künstler et al. 2016).

Plant defense processes are regulated by a complex, multilayered regulatory network in which defense-related plant hormones are playing pivotal roles (Aerts et al. 2021). The defense hormone salicylic acid (SA, 2-hydroxybenzoic acid) plays an important role in pathogen resistance of plants, and SA is considered to be mostly directed against biotrophic pathogens such as viruses (Vlot et al. 2009). However, SA is a multifaceted hormone that in addition to conferring defense responses in plants to pathogenic infections, also plays an important role in the development of abiotic stress tolerance (Horváth et al. 2007; Liu et al. 2022). SA was first reported to serve as an inducer of plant disease resistance in tobacco to tobacco mosaic virus (TMV) (White 1979). Initial studies have shown that TMV infection leads to an early accumulation of SA in inoculated leaves of TMV-resistant *Nicotiana tabacum* 'Xanthi NN'. High SA

levels were detected primarily in and around localized necrotic lesions (HR) that formed after TMV infection (Enyedi et al. 1992; Malamy et al. 1990). In the following years, several investigations confirmed the importance of SA in plant disease resistance (Delaney et al. 1994; Fodor et al. 1997; Gaffney et al. 1993; Klessig et al. 2018; Koo et al. 2020; Lee et al. 2016; Singh et al. 2004; Thomazella et al. 2021; Zhang and Li 2019). Lack of sufficient SA in infected plants leads to increased susceptibility to pathogens. For example, transgenic plants that express a gene encoding salicylate hydroxylase (*nahG*) are unable to accumulate an adequate amount of SA, which leads to suppressed plant defense responses and impaired disease resistance (Delaney et al. 1994; Gaffney et al. 1993; Künstler et al. 2019). A similar phenomenon can be observed in plants in which SA biosynthesis is inhibited (Pallas et al. 1996; Wildermuth et al. 2001; Zhang and Li 2019). SA can be stored in vacuoles of plant cells in various glycosylated forms like SA 2-*O*-β-D-glucoside (SAG) or salicylate glucose ester (SGE) (Ding and Ding 2020). Both SAG and SGE are considered to be inactive, stored forms of SA that can be hydrolyzed into free SA and transported from vacuoles to other parts of the plant cell (Vaca et al. 2017). Although the biological roles of the various stored forms of SA have not yet been fully explored, in planta free SA has various well-defined biological functions such as orchestrating plant defense to pathogens. SA-mediated signaling through the activation of nonexpressor of pathogenesis-related protein 1 (NPR1) leads to the coordinated induction of defense-associated genes encoding, among others, pathogenesis-related (PR) proteins (Chen et al. 2021; Murphy et al. 2020) and, ultimately, to the development of systemic acquired resistance that confers immunity to a wide spectrum of pathogens (Klessig et al. 2018).

In plants, the accumulation of reactive oxygen species (ROS) is one of the main defense responses to environmental stresses (e.g., drought, high/low temperatures, pathogenic infections). A certain level of in planta ROS can be considered normal, because of the constant activity of photosynthetic and respiratory electron transport chains. However, plants can actively produce ROS during both biotic stresses like pathogenic infections and abiotic stresses like drought, extreme temperatures, or herbicide exposure, often resulting in oxidative stress (Mittler et al. 2022). In plants that resist infections, higher ROS concentrations may promote PCD of infected plant cells (HR) and also the death or limitation of pathogens, including viruses, because of their high toxicity (Halliwell and Gutteridge 2015). On the other hand, at low concentrations, ROS play a crucial role in pathogen sensing and the activation of defense responses to pathogenic (viral) infections (Doke and Ohashi 1988; Hernández et al. 2016; Mittler et al. 2022). Because the accumulation of ROS may cause oxidative damage, different antioxidants are deployed in planta to avoid excessive oxidative stress. Most of the non-protein thiols in plants represent tripeptide glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine), an antioxidant that plays a pivotal role in the elimination of ROS (Hasanuzzaman et al. 2019). In addition to having an antioxidative role, GSH is also known as a central regulator of plant signaling during plant–pathogen interactions (Zechmann 2020). The positive correlation between GSH and plant disease resistance has been communicated in many papers (Großkinsky et al. 2012; Vanacker et al. 1999; Zechmann 2020), and increasing GSH content in plants by exogenous treatments induces disease resistance to various pathogens (Gullner et al. 1999; Künstler et al. 2020b). Interestingly the artificial elevation of GSH levels in tobacco by transgenic means also resulted in the elevation of SA levels, in parallel to enhanced disease resistance to viral, bacterial, and fungal pathogens (Ghanta et al. 2011, 2014; Künstler et al. 2019). Similarly, treatments with exogenous SA resulted in increasing GSH levels and enhanced viral resistance (Fodor et al. 1997; Mateo et al. 2006; Srivastava and Dwivedi 1998). Based on these results, the crosstalk between GSH and the SA-mediated defense pathway may significantly influence the outcome of plant disease resistance to various pathogens (Han et al. 2013; Kovacs et al. 2015; Künstler et al. 2020a).

Our previous research has shown that an interspecific tobacco hybrid (*Nicotiana edwardsonii* 'Columbia' [NEC]) displays enhanced resistance to virus-induced localized necrotic symptoms (HR) compared with another interspecific hybrid (*Nicotiana edwardsonii* [NE]) originating from the same parents (*N. glutinosa* and *N. clevelandii*) (Cole et al. 2001). NE is lacking two pairs of chromosomes ($n = 34$), whereas NEC has the full complement of chromosomes from both parents ($n = 36$). This genetic difference is a possible explanation for the significantly higher levels of both free and bound SA and the PR-1 protein in mature (at least 50 days old) NEC plants, even without any infection, compared with the control NE (Cole et al. 2004). The question arises whether NEC displays a bona fide viral resistance, that is, whether the enhanced resistance to HR-type necrosis is indeed accompanied by the inhibition of virus replication in case of infections by tobacco mosaic virus (TMV) and tobacco necrosis virus (TNV). Furthermore, we wanted to clarify whether high levels of SA indeed play a role in the viral resistance of NEC. Because GSH can influence the SA-mediated plant defense pathway, we also investigated whether elevated levels of SA in NEC might be coupled to higher than normal in planta GSH content, thereby contributing to the development of enhanced viral resistance. Finally, because SA-mediated resistance is also effective against other, non-viral pathogens and abiotic (oxidative) stress, we tested the response of NEC plants to incompatible (HR-inducing) and compatible bacterial pathogens and oxidative stress caused by methyl viologen (paraquat).

Results

To clarify that enhanced resistance to virus-induced localized necrotic symptoms in NEC is associated with the inhibition of virus replication, we monitored virus levels by two different methods. We assayed virion concentrations with antibodies specific for the coat protein of TMV and TNV using an enzyme-linked immunosorbent assay (ELISA) and monitored the accumulation of viral RNAs by amplifying the coding region of genes encoding the TMV and TNV coat proteins (*TMV CP* and *TNV CP*) with RT-qPCR. The appearance of necrotic symptoms caused by TMV and TNV was similar to that previously reported (Cole et al. 2004), that is, we observed much milder necrotic symptoms (localized lesions) on NEC plants compared with NE plants (Fig. 1). However, RT-qPCR revealed that TMV levels in NEC are only slightly, although significantly, lower than in NE, while TNV was barely detectable in NEC plants, as opposed to high TNV levels in NE (Fig. 1). Similar results were obtained with ELISA for both viruses. We concluded that TMV and TNV virus levels in NEC plants were significantly lower, as compared with NE (Fig. 1). Overall, we found that enhanced resistance to virus induced local necrotic symptoms in NEC is associated with significantly reduced levels of TMV and TNV, as compared with NE. In other words, NEC displays a bona fide resistance to viruses like TMV and TNV.

We have previously shown that NEC plants contain significantly higher levels of both free and especially bound SA, even without any infection, compared with the control NE (Cole et al. 2004). To determine whether high levels of SA indeed play a role in suppressing localized necrotic symptoms and virus levels, NEC plants were crossed with a transgenic tobacco (*N. tabacum* Xanthi NN *nahG*) unable to accumulate SA, because of overexpression of an SA-degrading enzyme, SA hydroxylase. Importantly, these SA-deficient plants display impaired

resistance to pathogens (Friedrich et al. 1995; Gaffney et al. 1993). The NEC × *N. tabacum* Xanthi NN nahG F_1 hybrids obtained were used to demonstrate how the absence of SA may influence virus-induced symptoms and virus replication. As expected, we found that in these TNV- and TMV-inoculated F_1 hybrids, free and bound SA both display the extremely low levels characteristic of nahG tobacco (Fig. 2). We have also confirmed our previous results demonstrating that in NEC plants infected with TNV and TMV, SA levels are indeed significantly higher, as compared with NE (data not shown; Cole et al. 2004). Importantly, the very low SA levels found in nahG × NEC F_1 hybrids were coupled to much more severe localized necrotic symptoms caused by virus inoculation, while virus levels were significantly elevated, as compared with NEC, for both TNV and TMV. In fact, symptom severity and virus levels in these F_1 hybrids were comparable to those found in SA-deficient nahG tobacco (Fig. 2).

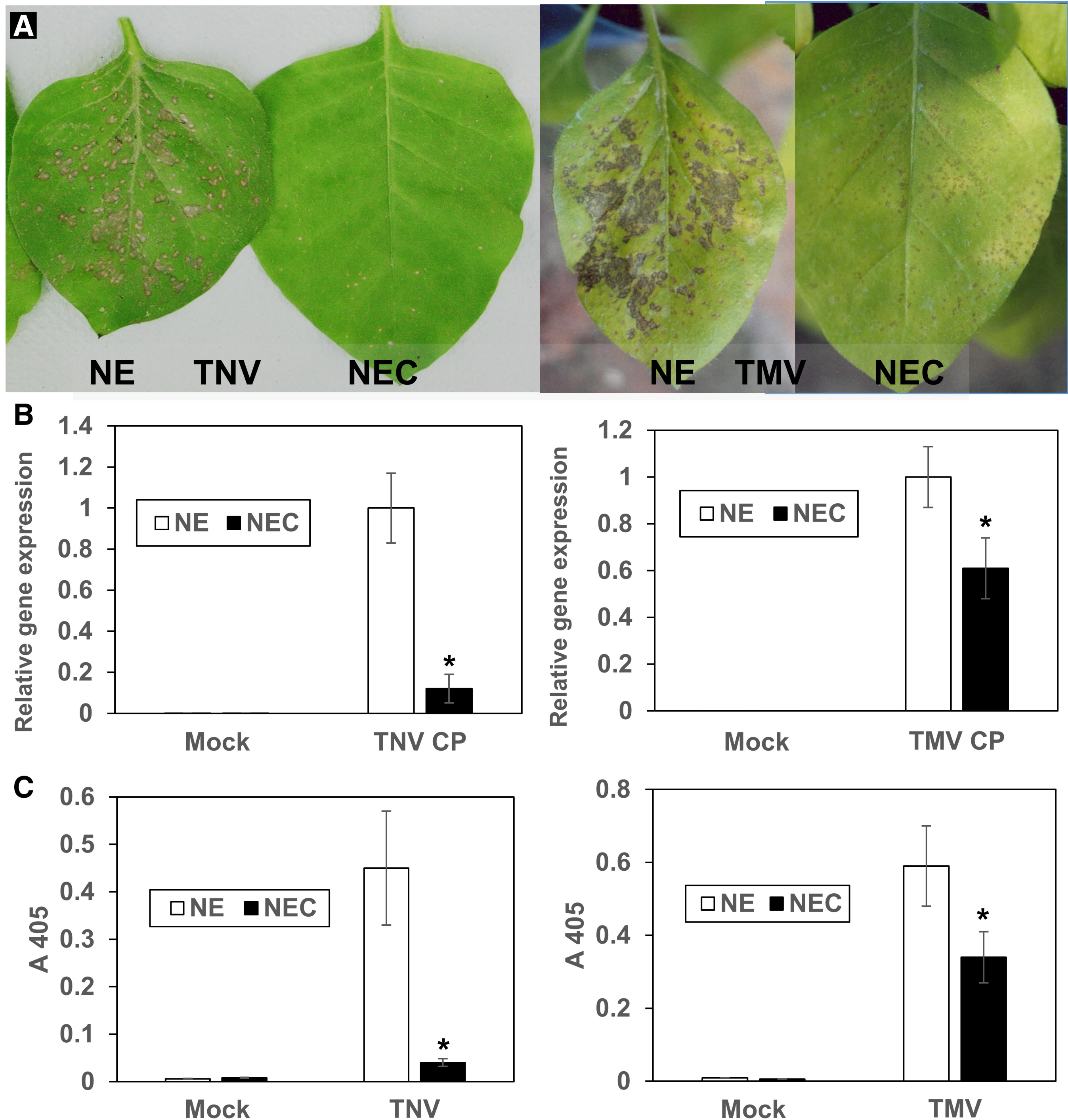

Fig. 1. Tobacco necrosis virus (TNV) and tobacco mosaic virus (TMV) symptoms (**A**), relative expression of TNV and TMV coat protein genes (*TNV CP* and *TMV CP*) (**B**), and TNV and TMV levels as detected by enzyme-linked immunosorbent assay (ELISA) (**C**) in *Nicotiana edwardsonii* (NE) and *Nicotiana edwardsonii* 'Columbia' (NEC). Virus symptoms were detected 5 days after inoculation. CP gene expression and the amount of CP was analyzed 3 days after inoculation. Absorbance values at 405 nm (A 405) were obtained from the plate reader after the addition of substrate. The bars show the average of three experiments. Error bars represent standard deviation. Asterisks (*) above bar graphs indicate statistically significant differences at $P \leq 0.05$ between NE and NEC.

As a control, wild-type *N. tabacum* 'Xanthi NN' (with normal SA levels) and its F_1 hybrid with NEC (Xanthi NN × NEC F_1) were also evaluated. The higher SA levels found in Xanthi NN × NEC F_1 hybrids relative to Xanthi NN were coupled to an intermediate phenotype of virus-elicited localized necrotic symptoms, while virus levels were only slightly, although significantly, elevated, as compared with NEC, for both TNV and TMV (Fig. 2). According to these results, elevated levels of SA are indeed necessary for controlling TMV- and TNV-induced localized necrotic symptoms and virus levels in NEC.

Because high SA levels play an important role in the enhanced viral resistance of NEC, we monitored the expression of different SA-related genes (*pathogenesis related-1*, *PR-1*; *salicylic acid glucosyltransferase*, *SAGT*) in the virus-infected plants. *PR-1* gene expression has long been used as a marker for SA-mediated disease resistance (Király et al. 2012; Klessig et al. 2018; Ward et al. 1991). According to our results, uninfected and untreated NEC plants display elevated expression of *PR-1*, as compared with NE (Fig. 3), a likely cause of constitutively high levels of PR-1 protein reported earlier in this host (Cole et al. 2004). However, elevated *PR-1* expression in NEC was also observed following mock inoculation and infection by TNV or TMV, indicating that the observed *PR-1* transcript accumulation is not virus specific (Fig. 3). Free SA can be converted into bound SA 2-*O*-β-D-glucoside mediated by SA glucosyltransferases (Enyedi et al. 1992; Hennig et al. 1993; Lee and Raskin 1999). As in the case of *PR-1*, expression of *SAGT* is also elevated in NEC, compared with NE, at 20 and 24 h after mock and virus (TMV and TNV) inoculation (Fig. 3). Mock inoculation significantly increased the expression of *SAGT* in NEC almost to the same extent as observed after TMV infection, as compared with NE. Interestingly, TNV infection increased the expression of *SAGT* even more as compared with both mock inoculation and TMV infection (Fig. 3).

It is known that SA and glutathione (GSH) are essential participants in plant resistance responses to different pathogens (Ghanta et al. 2011, 2014; Zhu et al. 2021). Therefore, monitoring GSH levels and the activity of GSH-related enzymes in the SA overproducer NEC tobacco may provide valuable information for elucidating the mechanism(s) of enhanced plant defense to TMV and TNV. Glutathione-*S*-transferases (GSTs) function in several stress response pathways, including plant defense to pathogens and abiotic stress, by using GSH as a substrate (Gullner et al. 2018; Mauch and Dudler 1993; Roxas et al. 2000). Therefore, we checked the expression of genes encoding plant-specific Tau and Phi class GST proteins (*GSTTau1* and *GSTPhi*) in NE and NEC plants inoculated with TMV or TNV at early time points (0, 6, 20, and 24 h after inoculation). Our results show that both TNV and TMV infection induced the expression of *GSTTau1* at 20 and 24 h after inoculation in NEC plants compared with NE plants (Figure 3). *GSTPhi* was induced 6 h after virus inoculation in both NE and NEC, but at later time points, the induced expression was observed only in NEC. However, mock inoculation also induced the expression of *GSTPhi* in NEC, indicating that the observed transcript accumulation is not entirely virus specific (Fig. 3).

In the next step, we monitored GST enzyme activity during TMV and TNV infection in NE and NEC plants. We observed significantly higher GST enzyme activity in NEC plants at several time points compared with NE controls (Fig. 4). Both TMV and TNV could significantly induce GST enzyme activity in NEC, but the effect of TMV inoculation on GST enzyme

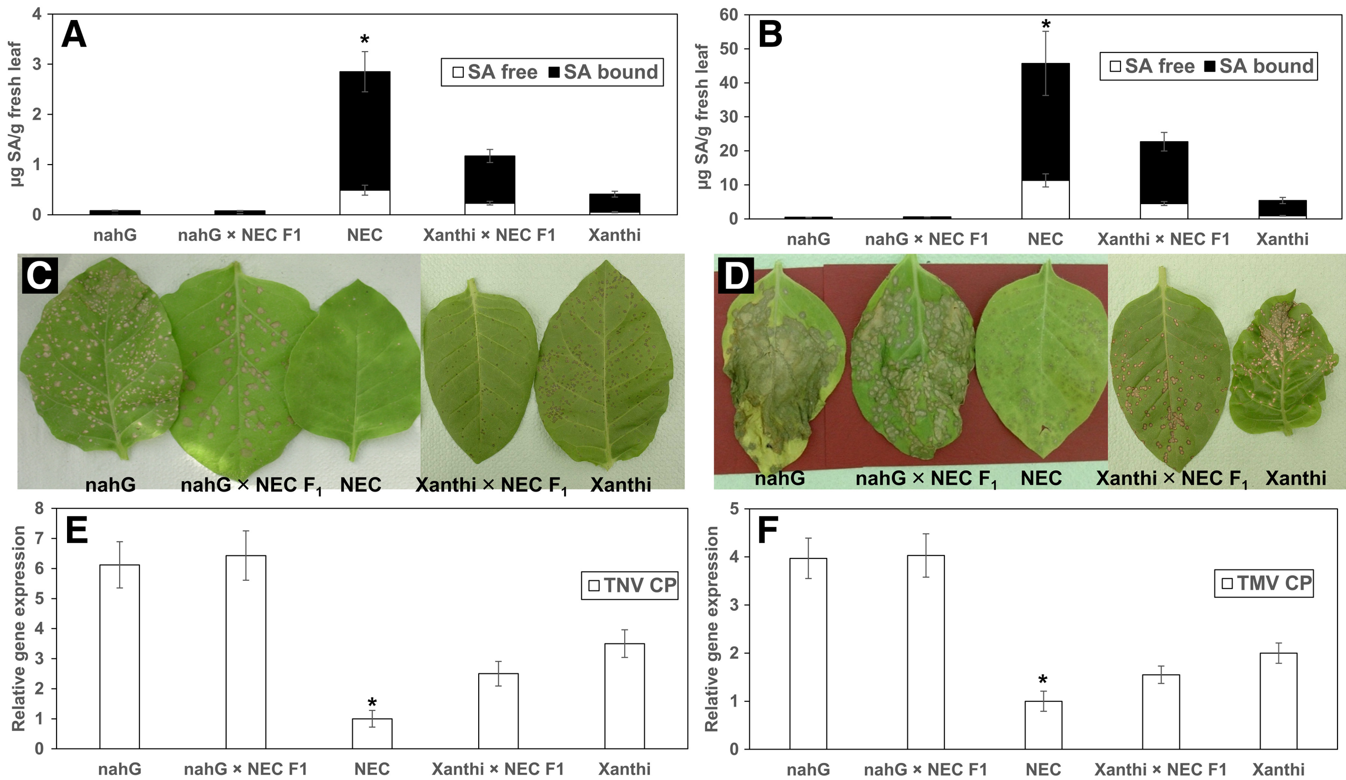

Fig. 2. Free and bound salicylic acid (SA) levels in response to inoculation by tobacco necrosis virus (TNV) (**A**) and tobacco mosaic virus (TMV) (**B**). Local necrotic symptoms (hypersensitive response [HR]) following TNV (**C**) and TMV (**D**) inoculation, and the relative expression of TNV and TMV coat protein genes (*TNV CP* and *TMV CP*) (**E and F**) in SA-deficient transgenic tobacco (*Nicotiana tabacum* 'Xanthi NN' nahG), *Nicotiana edwardsonii* 'Columbia' (NEC), and their F_1 hybrid (nahG × NEC F_1) in response to viral infections. As a control, wild-type *N. tabacum* Xanthi NN and its F_1 hybrid with NEC (Xanthi × NEC F_1) were also evaluated. Symptoms were analyzed 5 days after inoculation. *TNV CP* and *TMV CP* expression and free and bound SA levels were monitored 3 days after inoculation. Vertical bars represent the average of SA content and relative gene expression from three independent experiments. Error bars represent standard deviation. Asterisks (*) above bar graphs indicate statistically significant differences ($P \leq 0.05$) between NEC and all other genotypes.

activity was stronger and more persistent, as activity was higher in NEC at all investigated time points (Fig. 4). For this reason, in subsequent experiments, we focused primarily on studying the biochemical basis of enhanced NEC resistance to TMV infections.

Because we found that GST enzyme activity is higher in NEC plants as a result of TMV infection, and GST activity requires GSH, we also decided to assess the GSH content in NE and NEC plants. First, we determined the levels of low-molecular-weight thiols (LMW-thiols), because GSH is the most abundant LMW-thiol in plants (Hasanuzzaman et al. 2017). Our results showed that LMW-thiol levels are significantly increased in NEC compared with NE at 24, 48, and 96 h after TMV inoculation (Fig. 5A). Next, we investigated how GSH is distributed within different subcellular compartments in response to TMV infection. TMV inoculation significantly increased the GSH content in mitochondria (123%), chloroplasts (86%), nuclei (120%), and the cytosol (89%) of NEC mesophyll leaf cells, as opposed to NE, 3 days after inoculation (Fig. 5B). In summary, we have demonstrated that the enhanced resistance of NEC plants to TMV requires high levels of SA and is associated with an increase in cellular GSH content.

We wondered whether the enhanced resistance to viruses like TNV and TMV observed in NEC is also effective against other, non-viral pathogens and abiotic stresses. To answer this question, we inoculated NE and NEC plants with compatible (*Pseudomonas syringae* pv. *tabaci*) and incompatible (*Pseudomonas syringae* pv. *tomato* DC3000) bacterial pathogens. *P. syringae* pv. *tomato* DC3000 can be regarded as incompatible in NE and NEC plants, as it accumulated to almost an order of magnitude lower levels (10,000 to 30,000 CFU/cm^2) than *P. syringae* pv. *tabaci* (50,000 to 150,000 CFU/cm^2) (Fig. 6). NEC plants developed milder symptoms during both compatible and incompatible bacterial infections at 2 days after inoculation, as compared with NE controls (Figure 6). Furthermore, bacterial multiplication was strongly inhibited in NEC for both bacteria based on the number of CFU, as compared with NE plants (Fig. 6).

To investigate how these plants react to abiotic stress, we injected NE and NEC leaves with 25 and 50 μM aqueous solutions of methyl viologen (paraquat) to generate ROS (primarily superoxide). The infiltrated areas were outlined on the leaves, and the development of ROS-elicited tissue necrosis was assessed at 2 days after injection. Based on the size of the necrotized area, NEC plants proved to be more resistant to tissue necrosis caused by ROS, as the lesions were much smaller in size than those observed on NE plants at both concentrations (25 and 50 μM) of methyl viologen (Fig. 7). Overall, we have shown that the SA- and GSH-associated enhanced resistance of NEC plants is effective not only against TMV and TNV but also against bacterial pathogens and abiotic stress.

Discussion

The HR is one of the most studied forms of plant defense responses. HR comprises PCD localized to the pathogen entry point and associated with pathogen inhibition that frequently

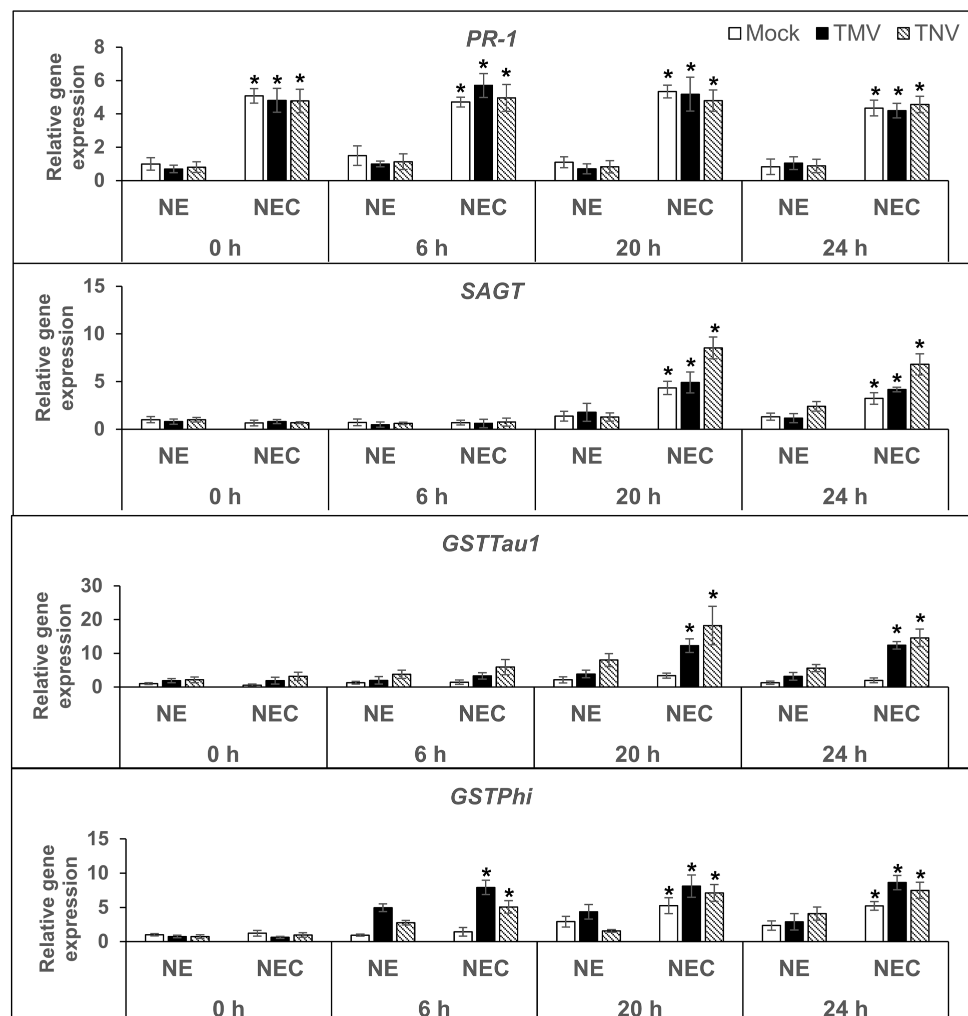

Fig. 3. Changes in the expression of different plant genes related to salicylic acid (SA) and glutathione metabolism (*pathogenesis related-1*, *PR-1*; *salicylic acid glucosyl-transferase*, *SAGT*; *glutathione S-transferase Tau1* and *Phi*, *GSTTau1* and *GSTPhi*) in *Nicotiana edwardsonii* (NE) and *Nicotiana edwardsonii* 'Columbia' (NEC) in response to tobacco necrosis virus (TNV), tobacco mosaic virus (TMV), and mock inoculation. Plants were sampled 0, 6, 20, and 24 h after inoculation. The bars show the average of three experiments. Error bars represent standard deviation. Asterisks (*) above bar graphs indicate statistically significant differences ($P \leq 0.05$) between NE and NEC within the respective time point and treatment.

results in visible localized tissue necrosis (Balint-Kurti 2019; Künstler et al. 2016). However, localized tissue necrosis associated with HR and the plant resistance response, although related, can be distinct processes, which means that necrotic symptom suppression is not necessarily connected to pathogen inhibition (Künstler et al. 2016; Mandadi and Scholthof 2013). We have previously shown that, in response to TMV and TNV, the interspecific tobacco hybrid NEC displays milder symptoms of HR-type tissue necrosis, as compared with another interspecific hybrid, NE, that originates from the same parents (*N. glutinosa* and *N. clevelandii*) (Cole et al. 2004). In this study, we have shown that the milder necrotic symptoms (localized lesions) in NEC plants are indeed correlated with the inhibition of TNV, manifested as drastically reduced TNV levels when detected by either RT-qPCR or ELISA, as compared with NE. However, TMV inoculation of NEC is also associated with a massive reduction of TMV-elicited HR-type symptoms, while virus levels are reduced only slightly, although significantly, as compared with NE. Our results imply that the appearance of milder localized necrotic symptoms in NEC does not necessarily mean a more effective virus inhibition. In fact, we have shown previously that exogenous treatments of tobacco leaves with superoxide-dismutase and catalase, resulting in an increased antioxidant capacity, causes a significant decline in HR-type necrosis following TMV inoculation, but virus levels do not change (Hafez et al. 2012).

The defense hormone SA plays a fundamental role in viral resistance by inhibiting viral replication, cell-to-cell movement, and systemic spread and by activating the small interfering RNA-mediated antiviral machinery in plants (Alamillo et al. 2006; Murphy et al. 2020; Peng et al. 2021; Vlot et al. 2009). We have shown previously that NEC plants contain significantly higher levels of both free and especially bound SA, even without any infection, as compared with the control, NE. The differences between NEC and NE became more pronounced following virus inoculation, as free SA levels were approximately doubled, and bound SA levels were 15-fold higher in NEC (Cole et al. 2004). Our present results demonstrate that high levels of SA in NEC are indeed necessary for the activation of enhanced plant defense, as F_1 hybrids derived from a cross between NEC and SA-deficient Xanthi NN nahG tobacco (NEC × nahG F_1) displayed significantly reduced SA levels along with a loss of viral resistance during TNV and TMV infection. However, in F_1 hybrids derived from a cross of NEC and wild-type (with normal SA levels) Xanthi NN tobacco (NEC × Xanthi F_1), SA levels intermediate to those in NEC and Xanthi NN were coupled to only a partial loss of enhanced viral resistance in response to TNV and TMV, underlining the role of SA in this enhanced viral defense phenotype. In line with our results, previous observations have shown that the absence of SA results in significantly impaired viral resistance (Baebler et al. 2014; Delaney et al. 1994; Künstler et al. 2019; Shadle et al. 2003). However, the in planta ratio of free and bound SA is also crucial, as high levels of free SA lead to increased pathogen resistance, but at the same time, the elevated free SA levels have a negative effect on plant growth and development (Koo et al. 2020; Peng et al. 2021). Interestingly, in NEC, the mild versus substantial increases in free versus bound SA, respectively, resulted in enhanced plant defense to TMV and TNV, as compared with NE, without altering plant growth and development. Recently, similar results were obtained in rice, in which a slight increase in free SA resulted in broad-spectrum resistance without a significant yield penalty (Liang et al. 2022; Zhang et al. 2022). Regarding bound SA, earlier research has indicated that the enzyme SA glucosyltransferase (SAGT) is activated in resistant tobacco in response to TMV infection (Enyedi and Raskin 1993). The function of SAGT is to convert free SA to bound (glycosylated) SA. Because SA is a defense hormone, its levels are strictly regulated in plants. SAGT is known to be activated not

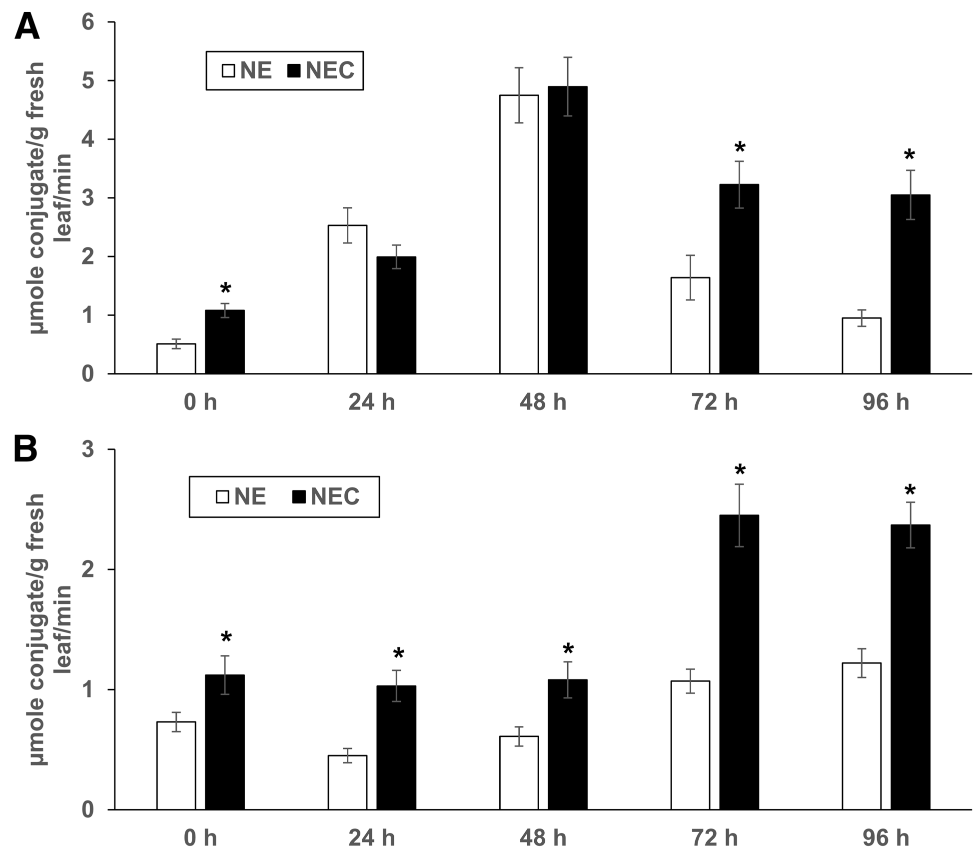

Fig. 4. Activity of glutathione *S*-transferase (GST) enzymes in *Nicotiana edwardsonii* (NE) and *Nicotiana edwardsonii* 'Columbia' (NEC) in response to tobacco necrosis virus (TNV) (**A**) and tobacco mosaic virus (TMV) (**B**) infections. GST activity was detected at 0, 24, 48, 72, and 96 h after inoculation. Vertical bars represent the average of GST activity from three independent experiments. Error bars represent standard deviation. Asterisks (*) above bar graphs indicate statistically significant differences ($P \leq 0.05$) between NE and NEC within the respective time points.

only by biotic but also by abiotic stresses. It has been shown that the expression of *SAGT* genes is induced in response to wounding stress in tobacco and rice (Seto et al. 2011). Moreover, mock inoculation (rubbing the leaf surface with silicon carbide particles) induced a transient increase in the amount of SA glucoside and SA glucose ester at 24 h after wounding in *Arabidopsis thaliana* (Ogawa et al. 2010). After viral inoculation, free SA levels increase substantially in resistant plants, and elevated free SA activates SAGT, resulting in the formation of bound SA (Fu and Dong 2013). Similar results were obtained in our present experiments, in which the expression of *SAGT* was induced in NEC at early time points after viral infection, especially in response to TNV. The possible cause of this phenomenon could be that TNV may induce a nonhost type of HR resistance in NE and NEC, as a systemic infection of any *Nicotiana* species by TNV is not known, except for *Nicotiana benthamiana* (Molnár et al. 1997). A nonhost type HR following TNV infection could be more responsive to the higher levels of free and bound SA present in NEC. In several *Arabidopsis* mutants, SA levels correlate well with the strength of nonhost resistance to bacterial and oomycete pathogens (Fonseca and Mysore 2019). Although the biological role of the different bound SA forms has not yet been fully explored, it can be assumed that the various bound SA forms stored in vacuoles are inactive but eventually hydrolyzed into active SA that can be exported to other parts of plant cells (Vaca et al. 2017). Importantly, the high levels of bound SA in NEC may significantly contribute to enhanced viral resistance, as the conversion of bound SA to free SA takes about 2 h (Hennig et al. 1993), while the de novo synthesis of SA following TMV inoculation takes about a day (Malamy et al. 1990).

SA-inducible PR genes and proteins may be reliable markers of pathogen resistance (Delaney et al. 1994; van Loon et al. 2006; Ward et al. 1991). However, the antiviral activity of these PR proteins has not been fully explored, although PR2a and PR3 proteins have been shown to decrease the infectivity of TMV in tobacco plants (Šindelářová and Šindelář 2005). In the present study, the constitutive expression of the SA marker gene *PR-1* found in NEC also points to the role of SA in plant defense to TMV and TNV, in line with our earlier results reporting high levels of the PR-1 protein, even in uninfected NEC (Cole et al. 2004). On the other hand, PR-1 proteins may not be functional as unique antiviral factors, as their overexpression in tobacco did not result in enhanced resistance to TMV (Cutt et al. 1989; Linthorst et al. 1989).

Previous research has demonstrated a positive correlation between the SA-mediated defense pathway and elevated GSH levels. Increasing amounts of SA in uninfected (healthy) plants are coupled to elevated GSH levels and vice versa, resulting in strengthened plant defense responses to various pathogens (Fodor et al. 1997; Ghanta et al. 2011, 2014; Han et al. 2013; Kovacs et al. 2015; Künstler et al. 2019, 2020b; Mateo et al. 2006). However, our results demonstrated that GSH is not elevated in mock-inoculated (i.e., uninfected) NEC compared with NE (this study), despite the high levels of SA found in NEC (Cole et al. 2004). Furthermore, there is no difference in levels of LMW-thiols (the majority of LMW-thiols being GSH) between NEC and NE at 0 h after TMV inoculation. We assume that the amounts of free (biologically active) SA in NEC positively affect GSH synthesis, while biologically inactive (stored) bound SA may have no significant direct effects on GSH levels. In fact, the majority of the high levels of SA in mock-inoculated NEC represent bound SA (Cole et al. 2004), a possible explanation for the similar amounts of GSH detected in mock-inoculated (uninfected) NEC and NE. Importantly, however, our results show that, following TMV inoculation, both GSH and LMW-thiol levels are significantly elevated in NEC plants compared with NE plants. This increase in GSH levels as a result of TMV infection is probably related to the several-fold increase in the amount of

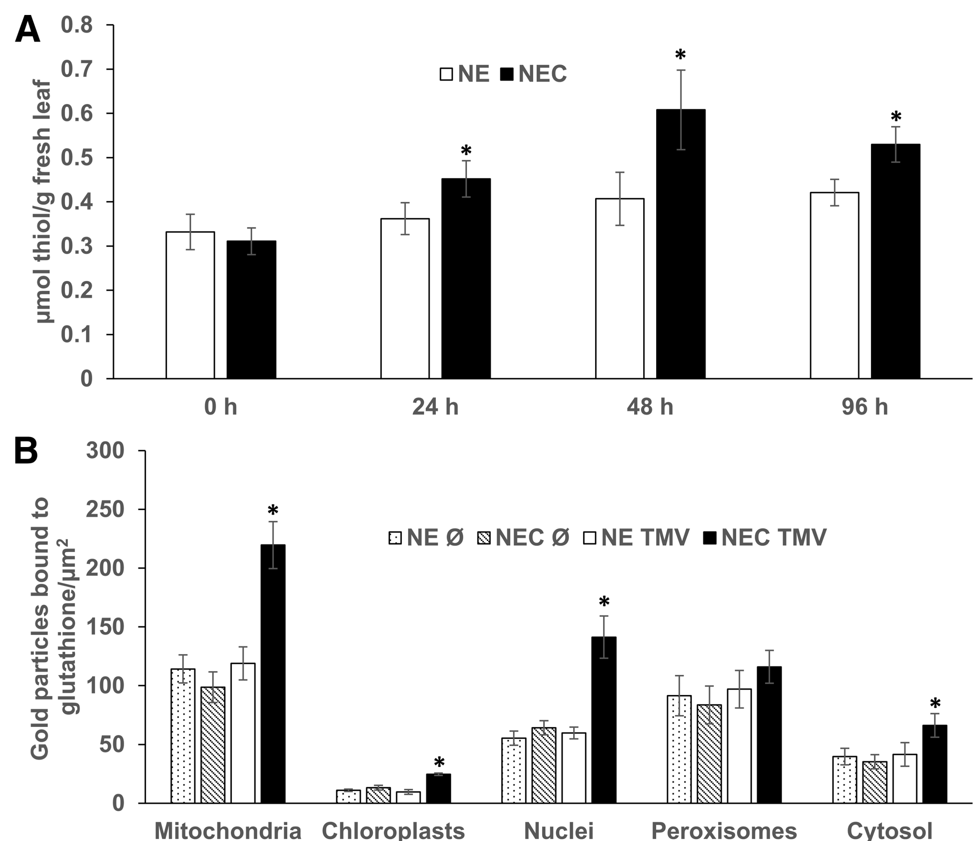

Fig. 5. A, Low-molecular-weight (LMW) thiol levels in tobacco mosaic virus (TMV)-inoculated *Nicotiana edwardsonii* (NE) and *Nicotiana edwardsonii* 'Columbia' (NEC) leaves detected at 0, 24, 48, and 96 h after inoculation. Vertical bars represent the average of LMW-thiol levels from three independent experiments. Error bars represent standard deviation. Asterisks (*) above bar graphs indicate statistically significant differences ($P \leq 0.05$) between NE and NEC within the respective time points. **B,** Subcellular distribution of glutathione in NE and NEC leaves 3 days after TMV inoculation. Mock-inoculated (Ø) samples were also taken at the same time. Vertical bars represent the average number of gold particles bound to glutathione per square micrometer in mesophyll leaf cells from three independent experiments. Error bars represent standard deviation. Asterisks (*) above bar graphs indicate statistically significant differences ($P \leq 0.05$) between different treatments within the respective cell compartments.

free SA in virus-infected NEC (Cole et al. 2004), likely reaching the threshold necessary for enhancing GSH synthesis and SA-mediated viral resistance. Because high in planta GSH levels are known to be associated with pathogen resistance (Großkinsky et al. 2012; Gullner et al. 1999; Künstler et al. 2020b; Vanacker et al. 1999; Zechmann 2020), it is likely that the elevated GSH levels detected in TMV-infected NEC also contribute to its enhanced defense to pathogens. The pivotal role of glutathione in SA-mediated plant viral resistance is also supported by our previous study demonstrating that in plants unable to accumulate SA, increased glutathione levels could compensate for the lack of SA, restoring TMV resistance (Künstler et al. 2019).

Elucidating the intracellular distribution of GSH in plants resistant to pathogenic infections may provide valuable information on possible mechanisms of defense. Our results showed that TMV infection is associated with significant increases in the levels of GSH in NEC at 3 days after inoculation in all cell organelles, except peroxisomes. Previously we have shown that the accumulation of GSH in mitochondria is dramatically elevated in a *Cucurbita pepo* cultivar resistant to zucchini yellow mosaic virus (ZYMV), as compared with a susceptible cultivar, resulting in milder virus symptoms and decreased virus titer (Zechmann and Müller 2008). Furthermore, the significant decrease of GSH in mitochondria of TMV-infected tobacco plants and *Botrytis cinerea*-infected *A. thaliana* plants was correlated with the accumulation of hydrogen peroxide in the cytoplasm, severe ultrastructural alterations, and the development of necrotic lesions (Király et al. 2012; Simon et al. 2013). In line with these results, NEC plants displayed a substantial increase of GSH levels in mitochondria in response to TMV inoculation, as compared with NE, associated with milder HR-type symptoms and reduced virus levels.

Plant chloroplasts are particularly sensitive to viral infections, as chloroplast structure and function are severely damaged in virus-infected plants, resulting in decreased photosynthetic activity and increased production of ROS (Li et al. 2016; Simon et al. 2013; Zechmann et al. 2021). Virus-induced enhanced ROS production in chloroplasts must be eliminated, and GSH as the most abundant non-enzymatic antioxidant plays an important role in this process (Zechmann 2020). For example, in susceptible plants, plum pox virus infection reduces the activity of the ascorbate–glutathione cycle enzymes coupled to increased lipid peroxidation and the accumulation of one of the major ROS, hydrogen peroxide (Hernández et al. 2006). In addition, we have shown previously that strongly elevated amounts of GSH in chloroplasts of resistant *Cucurbita pepo* plants infected with ZYMV were related to the absence of symptoms (Zechmann and Müller 2008). In fact, enhanced accumulation of GSH in chloroplasts may also participate in the elimination of virus-induced oxidative damage and milder necrotic (HR) symptoms in NEC plants. The importance of GSH and other antioxidants in preserving plant chloroplast integrity is also emphasized by a recent study, showing that the γb protein of barley stripe mosaic virus disrupts the antioxidant defense system of chloroplasts to confer efficient virus replication (Wang et al. 2021).

During pathogen attack, defense signals processed in the cytosol activate defense gene expression in nuclei. Transcripts of different defense genes are then transported from the nuclei into the cytosol and other organelles, and GSH has a substantial impact on translation and posttranslational modification of proteins (Cheng et al. 2015; Corpas et al. 2022). Furthermore, GSH has a significant influence on signaling and regulation of defense proteins in plants through redox modifications (Mata-Pérez and Spoel 2019; Mittler et al. 2022). It has been proposed that GSH functions as a redox sensor in nuclei by providing the appropriate redox environment for DNA replication (García-Giménez et al. 2013). We have previously reported that in TMV-infected, resistant tobacco plants, nuclei show a slight increase of GSH (Király et al. 2012). Furthermore, a strong increase of GSH in nuclei and the cytosol was correlated with suppressed symptom development and virus content in susceptible tobacco plants infected with TMV (Höller et al. 2010). In line with these results,

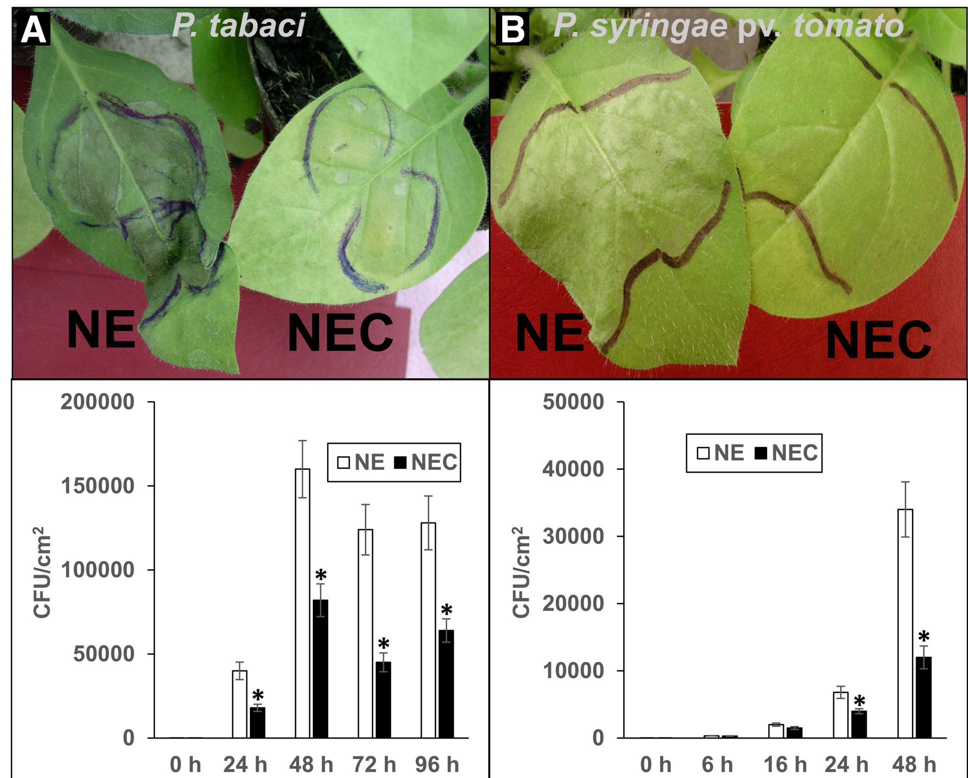

Fig. 6. Symptom development and bacterial multiplication in *Nicotiana edwardsonii* (NE) and *Nicotiana edwardsonii* 'Columbia' (NEC) during compatible (**A**) and incompatible (**B**) bacterial infections caused by *Pseudomonas tabaci* and *Pseudomonas syringae* pv. *tomato* DC3000, respectively. Circled areas in plant leaves were syringe inoculated with bacterial suspensions (7×10^5 CFU/ml), and symptom development was evaluated 2 days after inoculation. Bacterial multiplication was evaluated during compatible interactions at 0, 24, 48, 72, and 96 h after inoculation and during incompatible interactions at 0, 6, 16, 24, and 48 h after inoculation. Vertical bars represent average bacterial concentrations (CFU/cm^2) from three independent experiments. Error bars represent standard deviation. Asterisks (*) above bar graphs indicate statistically significant differences ($P \leq 0.05$) between NE and NEC within the respective time points.

we report here that significantly elevated amounts of GSH in the nuclei and the cytosol of NEC are associated with enhanced resistance to TMV.

SA is a key signaling molecule involved in orchestrating resistance not only to viruses but also to bacterial and fungal pathogens and abiotic stresses. To elucidate how elevated levels of SA in NEC may influence resistance to bacterial pathogens, we inoculated plants with two different phytopathogenic bacteria. According to our results, NEC plants developed milder symptoms and reduced bacterial multiplication during both compatible (*P. syringae* pv. *tabaci*) and incompatible (*P. syringae* pv. *tomato* DC3000) interactions compared with the NE control. In agreement with our results others have shown that *Arabidopsis* hybrids with elevated amounts of free and glycosylated SA show enhanced resistance to *P. syringae* pv. *tomato* DC3000 (Yang et al. 2015). The pivotal role of SA in pathogen resistance is also underlined by the fact that certain plant pathogenic bacteria actively hinder the accumulation of SA in the plant host by employing different methods (Qi et al. 2018). For example, '*Candidatus* Liberibacter asiaticus', the bacterial pathogen responsible for citrus greening, suppresses plant defense by encoding an SA hydroxylase, thus inhibiting SA accumulation and allowing successful infection (Li et al. 2017).

SA-mediated defense responses are effective not only against pathogens (biotic stresses) but also in limiting abiotic stresses (Fuerst and Vaughn 1990; Horváth et al. 2007). Furthermore, plants may actively produce ROS during both pathogenic infections and abiotic stresses, including drought or herbicide exposure, often resulting in oxidative stress (Mittler et al. 2022). The herbicide paraquat (methyl viologen) blocks photosynthesis by accepting electrons from photosystem I, which will then transfer electrons to O_2 to produce the ROS superoxide (O_2) (Yoon et al. 2011). The enhanced production of superoxide induces oxidative stress in paraquat-treated plants. One way to induce paraquat tolerance in plants is by enhancing antioxidant capacity, resulting in a significant reduction in the amount of ROS (Barna et al. 1993; Fuerst and Vaughn 1990; Furusawa et al. 1984). In fact, elevating SA levels in barley by exogenous treatments resulted in increased antioxidant capacity in leaves exposed to paraquat, while SA deficiency in tobacco confers a compromised antioxidant status (Fuerst and Vaughn 1990; Király et al. 2002). In line with these findings, our results also show that elevated SA levels in NEC induce paraquat tolerance, as compared with NE, manifested as the appearance of smaller necrotic lesions at the site of paraquat infiltration. It seems that the paraquat tolerance of NEC could be associated with an enhanced antioxidant status provided by enzymes like GSTs. GSTs are stress-inducible enzymes that may also have antioxidant

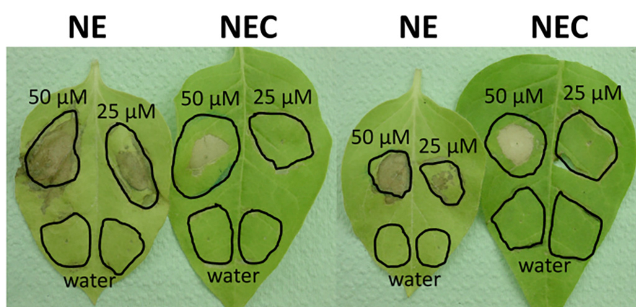

Fig. 7. Methyl viologen (paraquat)-induced tissue necrosis symptoms in *Nicotiana edwardsonii* (NE) and *Nicotiana edwardsonii* 'Columbia' (NEC) leaves 2 days after infiltration. Circled areas in plant leaves were injected with 25 and 50 μM aqueous solutions of methyl viologen. Control inoculations were performed with tap water. Photographs are from one representative experiment; the experiment was repeated three times with similar results.

(glutathione peroxidase [GPX] or dehydroascorbate reductase) activity (Gullner et al. 2018; Horváth et al. 2020). In fact, elevated GST activity in tobacco was correlated with paraquat tolerance, and overexpression of a cotton GST exhibiting GPX activity conferred enhanced resistance to paraquat in tobacco (Gullner et al. 1991; Yu et al. 2003). Therefore, the increased expression of *GSTTau1* and *GSTPhi* that we have detected in virus-infected and mock-inoculated NEC plants may contribute not only to enhanced pathogen resistance but also to elevated plant antioxidant defenses and paraquat tolerance in NEC. Our results imply that one common mechanism that confers enhanced resistance of NEC to both biotic and abiotic stresses is the enhanced resistance to oxidative damage likely manifested as an elevated plant antioxidant capacity. It seems that during incompatible plant–virus interactions (HR), NEC responds with chlorosis/milder necrosis (as opposed to full necrosis), and this response is not specific to viral resistance, as it is also apparent following incompatible or compatible bacterial infections. Furthermore, our experiment demonstrating the enhanced paraquat tolerance of NEC strongly suggests that the suppressed necrotic symptoms in response to pathogenic infections and herbicide stress may be related to ROS accumulation, which is likely controlled by elevated antioxidant activity, including that of GST isoenzymes. The enhanced resistance of NEC to necrotic symptoms resembles that of tobacco (*N. tabacum*) plants with an elevated antioxidant capacity (including enhanced GST activity) that are tolerant not only to paraquat stress but also to necrosis caused by viral, bacterial, and fungal pathogens (Barna et al. 1993; Gullner et al. 1991). However, these paraquat-tolerant tobaccos are not resistant to pathogens per se, as smaller necrotic areas during a bacteria-induced HR do not correlate with decreased levels of *P. syringae* pv. *syringae* (Ádám et al. 1990). Similarly, tobacco plants displaying an elevated antioxidant capacity following treatments with low concentrations of ROS (5 to 10 mM hydrogen peroxide) responded to incompatible viral (TMV) and bacterial (*Pseudomonas syringae* pv. *phaseolicola*) infections with suppressed HR-type necrotic symptoms, while pathogen levels remained unchanged (Hafez et al. 2012). Therefore, it is likely that the combined resistance of NEC to both abiotic (oxidative) and biotic stresses is facilitated not only by an elevated antioxidant capacity (and controlled ROS levels) but also by an interplay between SA and GSH levels. It is known that increased amounts of SA in uninfected plants result in elevated GSH levels and vice versa, leading to enhanced resistance to viral, bacterial, and fungal pathogens (Fodor et al. 1997; Ghanta et al. 2011, 2014; Künstler et al. 2019, 2020b).

In summary, our results collectively show that an interspecific tobacco hybrid, NEC, displays enhanced resistance to TMV and TNV, compared with another hybrid, NE, originating from the same parents. Enhanced viral resistance is manifested as both milder local necrotic (HR) symptoms and reduced virus titer. The presence of an adequate amount of SA is required for the enhanced viral resistance of NEC, because F_1 hybrids derived from a cross between NEC and SA-deficient nahG tobacco (NEC × nahG F_1) display significantly reduced SA levels coupled to seriously impaired viral resistance. We have detected elevated levels of GSH evident in most subcellular compartments in NEC in response to viral infection, suggesting that GSH also contributes to the strengthened viral resistance of NEC. Furthermore, NEC displays an enhanced resistance not only to viral pathogens but also to bacterial infections and abiotic (oxidative) stress induced by paraquat treatments.

We have developed a theoretical model (working hypothesis) based on the results of our study to explain how the interplay of SA and GSH may contribute to the enhanced resistance of NEC to both biotic and abiotic (oxidative) stresses (Figure 8). In brief, virus (TMV) infection of NEC results in elevated SA levels,

leading to an accumulation of ROS and GSH and induction of GSTs. In turn, GSH mediates SA and ROS signaling through a positive feedback loop, sustaining enhanced biotic and abiotic (oxidative) stress resistance in NEC. SA- and GSH-mediated plant defense signaling may also contribute to the enhanced viral resistance of NEC through elevated expression of PR genes and enhanced RNA-dependent RNA polymerase 1 (RDR1) activity via the action of Nonexpressor of PR1 (NPR1).

Materials and Methods

Plant materials, pathogen inoculation, and paraquat treatment

The following tobacco plants were used in our experiments: *Nicotiana edwardsonii* (NE) and *Nicotiana edwardsonii* Columbia (NEC) interspecific tobacco hybrids (Chrisite 1969; Cole et al. 2001), *Nicotiana tabacum* Samsun *nn*, *Nicotiana tabacum* Xanthi NN (wild type), *Nicotiana tabacum* Xanthi NN nahG (SA-deficient transgenic nahG line) (Gaffney et al. 1993), F_1 hybrid plants derived either from crosses of nahG tobacco and NEC (nahG × NEC F_1) or crosses of wild-type Xanthi NN tobacco and NEC (Xanthi × NEC F_1). Crosses were done by mechanical pollination (hand pollination). First, we used nahG and wild-type Xanthi NN tobacco as female (♀) and NEC as male (♂) parents. Reciprocal crosses were also performed, and the F_1 plants obtained gave similar experimental results. Presence or absence of expression of the *nahG* transgene in F_1 progeny was verified by RT-qPCR (data not shown). Tobacco plants were grown on commercial garden soil under greenhouse conditions with standard parameters (temperatures between 20 and 23°C, approximately 16 h of daylight with daily watering).

TMV U1 strain and TNV DEH strain (Bacsó et al. 2016) were used as viral pathogens. Both TMV and TNV were maintained in a susceptible host, *N. tabacum* Samsun *nn*. For TMV, young, systemically infected upper leaves showing typical mosaic symptoms were used for inoculation, while locally infected leaves showing necrotic lesions (HR) were used for TNV inoculation. One gram of virus-containing leaves was homogenized in 10 ml of tap water with silicon carbide (carborundum) powder as an abrasive. The third or fourth true leaves (counted from the bottom to the top) of 50- to 60-day-old NE and NEC plants were inoculated mechanically. Symptoms were visually evaluated 3 days after inoculation on inoculated leaves. Mock inoculations with tap water and silicon carbide powder were also performed as a control.

Plant pathogenic bacteria (*Pseudomonas syringae* pv. *tabaci* H10 and *P. syringae* pv. *tomato* DC3000) were maintained on King's B medium at 26 to 28°C. For bacterial inoculation, 1-day-old liquid cultures derived from a single bacterial colony were used with a final inoculum concentration of 7×10^5 CFU/ml adjusted with a spectrophotometer (CO8000 cell density meter, WPA, Cambridge, U.K.). The inoculum was infiltrated into interveinal leaf segments of NE and NEC on the adaxial leaf side with a hypodermic syringe and needle. Symptom development was evaluated at 2 days after inoculation.

NE and NEC leaves were infiltrated with 25 and 50 μM aqueous solutions of methyl viologen (paraquat) to test how these plants react to abiotic stresses. The infiltrated areas were marked on the leaves, and paraquat-induced tissue necrosis on NE and NEC leaves was evaluated 2 days after infiltration based on the size of the necrotized area. Control infiltrations were also performed by using tap water.

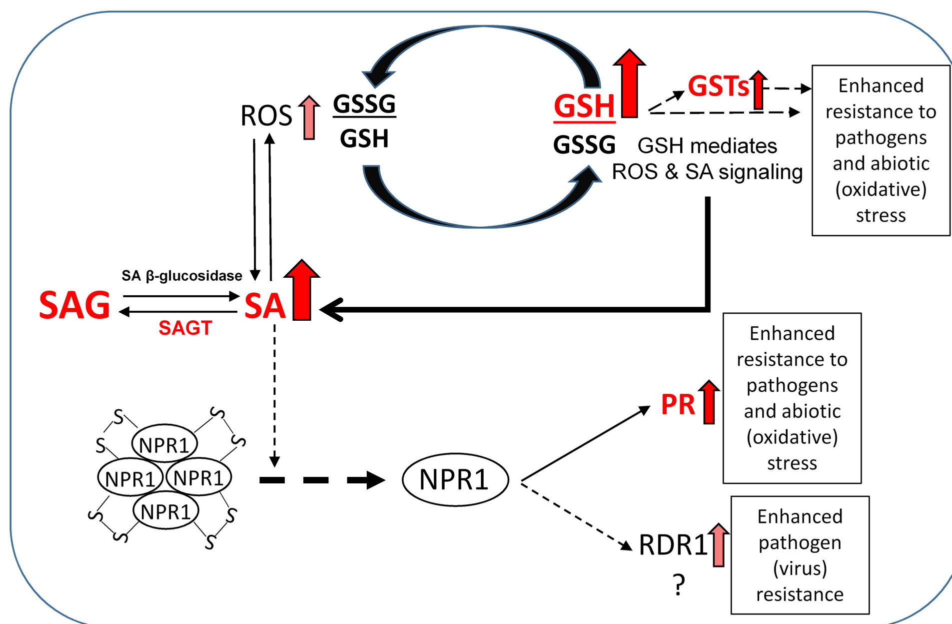

Fig. 8. A possible model of enhanced resistance of *Nicotiana edwardsonii* 'Columbia' (NEC) to pathogens (biotic stress) and abiotic (oxidative) stress. Biotic and abiotic stresses in NEC rapidly elevate levels of free salicylic acid (SA) by releasing SA from bound salicylic acid (SAG) pools abundant in NEC. SA accumulation is mediated by SA β-glucosidase, while SA glucosyl transferase (SAGT) is responsible for SAG synthesis (Enyedi and Raskin 1993; Fu and Dong 2013; Yao et al. 2007). The elevation in free SA levels associated with different stresses increases reactive oxygen species (ROS) production, while ROS may also induce SA (Chen et al. 1993; Durner and Klessig 1995; Klessig et al. 2018). ROS formation during stress modulates the ratio of reduced/oxidized glutathione (GSH/GSSG) and ultimately increases GSH levels associated with the induction of glutathione *S*-transferases (GSTs), resulting in enhanced resistance to pathogens and abiotic (oxidative) stress (Gullner et al. 2018). Furthermore, GSH may mediate the accumulation of SA, possibly conferring a self-amplification of defense responses (Ghanta et al. 2011, 2014; Han et al. 2013; Künstler et al. 2019). SA- and GSH-mediated plant defense signaling may also contribute to enhanced biotic and abiotic stress resistance through the elevated expression of pathogenesis-related (*PR*) genes via the oligomer to monomer conversion of Nonexpressor of PR1 (NPR1) (Liu et al. 2002; Murphy et al. 2020). It is also possible that elevated RNA-dependent RNA polymerase 1 (RDR1) activity mediated by NPR1 may participate in the SA-induced enhanced pathogen (virus) resistance of NEC (Lee et al. 2016; Murphy et al. 2020). Components of the model based on results of the present study are depicted in red.

Monitoring virus accumulation and plant defense gene expression

To analyze virus levels in plant leaves, we employed two methods: (i) assaying virion concentrations with antibodies specific for the coat proteins of TMV and TNV using an ELISA; and (ii) detecting the accumulation of viral RNAs by amplifying the coding region of TMV and TNV coat protein genes (*TMV CP* and *TNV CP*) with RT-qPCR.

To determine viral concentrations, double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) from virus-inoculated tobacco leaves (sampled 3 days after inoculation) were performed by using a TMV-ELISA kit (Bioreba AG, Reinach, Switzerland) and a TNV-E ELISA kit (Loewe Biochemica GMBH, Sauerlach, Germany) according to the manufacturers' instructions. Briefly, 96-well polystyrene microplates were filled with TMV or TNV antibodies (immunoglobulin G [IgG]) diluted 1:1,000 in coating buffer (pH 9.6) and incubated at 37°C for 3 to 4 h. Following several washes with tap water and PBS-Tween (pH 7.4), wells were filled with leaf homogenate (sampled 3 days after virus inoculation) diluted 1:20 in PBS extraction buffer (PBS-Tween + 0.2% polyvinylpyrrolidone [PVP] + 0.2% bovine serum albumin [BSA]) and incubated overnight at 4°C. Following repeated washes, wells were filled with alkaline phosphatase-conjugated IgG (diluted 1:1,000 in PBS extraction buffer) and incubated at 37°C for 2 to 4 h. Finally, *p*-nitrophenyl phosphate at 1 mg/ml (substrate) was added to the washed wells, which were incubated at 37°C for 10 to 15 min. Absorbance values were read by a Multiskan ELISA plate reader (Thermo Scientific, Waltham, MA, U.S.A.) at 405 nm.

We also determined virus levels and plant defense gene expression with RT-qPCR. To determine virus levels, inoculated leaves were sampled 3 days after inoculation. To analyze plant defense gene expression, virus-inoculated leaves were sampled at early time points after inoculation (0, 6, 20, and 24 h after inoculation). Frozen leaves were homogenized with liquid N_2 and 100 mg ground leaf tissue was used for total RNA extraction using a Plant Total RNA Extraction Miniprep System Kit according to the manufacturer's instructions (Viogen BioTek, New Taipei City, Taiwan). Following RNA isolation, DNAse treatment was performed with RQ1 RNase-Free DNase (Promega, Madison, WI, U.S.A.). RNA quantity and quality were assessed by a spectrophotometer (Maestrogen, Hsinchu City, Taiwan), and RNA degradation was also checked by formaldehyde agarose gel electrophoresis of total RNA. One thousand nanograms total RNA was used for reverse transcription (RT) in each sample. RT was conducted with a RevertAid^{TM} H^- cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.) according to manufacturer's instructions using reverse primers specific for either TMV or TNV coat protein genes (*TMV CP* and *TNV CP*) and an oligo-dT primer. An RT-negative control (sample with RNA but without reverse transcriptase) was used during reverse transcription. Quantitative PCR for assaying virus coat protein gene (*TMV CP* or *TNV CP*) and plant defense gene expression was conducted with the 2x SYBR FAST qPCR Master Mix reagent supplied by KAPA Biosystems (Wilmington, MA, U.S.A.), using 2.5 µl of template cDNA (20-fold diluted) in a total reaction volume of 15 µl (Künstler et al. 2019). PCR amplifications were carried out in a CFX-96 real-time thermocycler (Bio-Rad, Hercules, CA, U.S.A) by using a standard program based on the manufacturer's recommendations: 95°C for 2 min, 40 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. This was followed by a melting-curve analysis from 65 to 95°C at increments of 0.5°C to determine amplicon specificity. All primer pairs gave a single melting-curve peak and a single distinct band on agarose gels, with a size corresponding to predicted amplicon lengths, indicating sufficient specificity of primers and amplicons. All reactions were performed with three technical replicates per biological sample. Gene expression was normalized to a tobacco actin (*Act*) gene as a reference. Suitability of *Act* as a reference gene was tested by cycle threshold variation analysis in response to virus (TNV and TMV) and mock inoculations. Significant changes in cycle threshold values (mean ± SD) for *Act* were not observed in response to inoculations. Relative gene expression was calculated by employing the comparative C_T ($2^{-\Delta\Delta CT}$) method (Schmittgen and Livak 2008). Primer efficiencies were between 97 and 100% for the genes tested. Oligonucleotide primers used in these experiments are listed in Table 1. Primers were designed by us with the aid of the Primer Premier 5 program (PREMIER Biosoft International, San Francisco, CA, U.S.A), except for *GSTTau1* and *GSTPhi* (Juhász and Gullner 2014).

Determination of bacterial multiplication

Numbers of viable bacteria in tobacco leaves infected with *P. syringae* pv. *tabaci* and *P. syringae* pv. *tomato* were monitored by a modified serial dilution method (Ott et al. 2006). In brief, leaf samples were taken at 0, 24, 48, 72, and 96 h after inoculation in case of compatible interactions when the plants were infected with *P. syringae* pv. *tabaci.* During incompatible interactions (*P. syringae* pv. *tomato*) samples were taken at 0, 6, 16, 24, and 48 h after inoculation. Eight leaf disks (1 cm^2 each) per sample were taken with a cork borer from infiltrated NE and NEC leaf areas. Surface-sterilized leaf disks were homogenized in 1 ml of potassium-phosphate buffer (10 mM, pH 7.0). Serial dilutions (10×, 100×, and 1,000×) were plated on King's B agar medium in Petri dishes (10 µl/dilution, at least six dilutions/Petri dish). Colony numbers per plated dilution were used to calculate the original numbers of bacterial cells present in 1 cm^2 of inoculated leaf tissue.

Determination of free and bound SA

Free and bound forms of SA were detected by high-performance liquid chromatography (HPLC) analysis. The third true leaves (counted from the bottom to the top) of 50- to 60-day-old, TMV- or TNV-inoculated NEC, Xanthi NN wild-type, and SA-deficient nahG tobacco and crosses of tobacco and NEC (Xanthi × NEC F_1 and nahG × NEC F_1 hybrids) were sampled to detect free and bound SA forms at 3 days after virus inoculation, using ortho-anisic acid as an internal standard (Meuwly and Metraux 1993). For each sample 1 g of tobacco leaf tissue was ground in quartz sand and liquid nitrogen. The ground tissue was extracted with 70% methanol. Bound SA was hydrolyzed with 4 M hydrochloric acid. The following steps of the extraction were carried out as described in detail by Cole et al. (2004) and Pál et al. (2005). The separation of SA was performed by HPLC. Just before HPLC analysis, the evaporated samples were resuspended in 500 µl of the HPLC starting mobile phase. SA was quantified fluorimetrically (W474 scanning fluorescence detector, Waters, Milford, MA, U.S.A.), with excitation at 305 nm and emission at 407 nm.

Determination of GST enzyme activity

For the detection of GST enzyme activity, 0.5 g NE and NEC leaf materials (sampled at 0, 24, 48, 72, and 96 h after virus inoculation) were homogenized in cold homogenizing buffer (3 ml of 50 mM TRIS buffer [pH 7.8] containing 1 mM ethylenediaminetetraacetic acid disodium salt [EDTA-Na_2] and 7.5% polyvinylpyrrolidone) at 0 to 4°C. The homogenates were centrifuged (12,000 rpm, 20 min, 4°C), and GST enzyme activity was measured in the supernatant spectrophotometrically. GST activity was determined by assaying the formation of the conjugate reaction product of glutathione and 1-chloro-2,4-dinitrobenzene as the substrate at 340 nm (Mauch and Dudler 1993).

Spectrophotometric assay of acid-soluble non-protein thiols

To detect acid-soluble non-protein thiols, NE and NEC leaves were sampled at different time points (0, 24, 48, and 96 h) after virus inoculation. Leaves were pulverized in a mortar with liquid N_2 and homogenized in 5 ml of cold homogenizing solution (6.099 g 5-sulphosalicylic acid, 0.45 g ascorbic acid, and 0.1116 g EDTA-Na_2 dissolved in 300 ml of distilled water). The suspension was centrifuged for 20 min at 10,000 × *g* (4°C), and the supernatant was used for spectrophotometric assays applying 5,5′-dithio-bis-(2-nitrobenzoic acid) as a derivatizing agent for thiol groups. Absorbance was assayed at 412 nm. To calculate thiol levels, a calibration curve was prepared with glutathione solutions of known concentrations (De Kok and Graham 1989).

Subcellular detection of glutathione by electron microscopy

Preparation of samples for transmission electron microscopy and immunogold labeling of glutathione (including negative controls) was done with ultrathin sections on nickel grids, as previously described (Zechmann and Müller 2010; Zechmann et al. 2006). Samples of the youngest fully developed leaves were fixed in 2.5% paraformaldehyde and 0.5% glutardialdehyde in 0.06 M phosphate buffer (pH 7.2) for 90 min. Samples were rinsed in buffer and dehydrated in increasing concentrations of acetone (50, 70, and 90%) and were infiltrated with increasing concentrations of LR-White resin (30, 60, and 100%; London Resin Company, Berkshire, U.K.). Samples were finally embedded in LR-White resin and were polymerized at 50°C for 48 h in small plastic containers. Ultrathin sections of the samples were blocked with 2% BSA in phosphate-buffered saline (PBS, pH 7.2) and were subsequently treated for 2 h at room temperature with primary antibodies (anti-glutathione rabbit polyclonal IgG, Millipore, Billerica, MA, U.S.A.) diluted 1:50 (glutathione antibody) in PBS containing 1% goat serum. After a short rinse in PBS, samples were incubated for 90 min at room temperature with a 10-nm gold-conjugated secondary antibody (goat anti-rabbit IgG; British BioCell International, Cardiff, U.K.) diluted 1:50 in PBS. After a short wash in PBS and distilled water, labeled grids were observed in a Philips CM10 transmission electron microscope. At least four different samples from mock-inoculated and TMV-infected leaves were used for statistical evaluation. A minimum of 20 (peroxisomes and vacuoles) to 60 (other cell structures) sectioned cell structures of at least 15 different cells per biological experiment were analyzed for gold particle density. For this purpose, micrographs of randomly photographed immunogold-labeled sections were digitalized. Individual cell structures (cytosol, mitochondria, nuclei, peroxisomes, plastids, and the cytosol) were manually traced, and gold particles were automatically counted using the software package Cell D with the particle analysis tool (Olympus, Life and Material Science Europa, Hamburg, Germany). Unspecific background labeling was determined in all samples at or below 0.1 gold particles/μm^2 by counting gold particles on 30 different areas outside the specimen. Background was subtracted from the values obtained inside the samples.

Statistical analysis

All analyses were performed using three independent biological experiments with at least three technical replicates per biological sample. Statistical analyses were conducted using the Statistica 13 software (TIBCO Software, Palo Alto, CA, U.S.A.). For statistical analysis, analysis of variance followed by Tukey's post hoc test was used, except for subcellular glutathione determination, for which the number of gold particles per square micrometer was analyzed with the nonparametric Kruskal-Wallis test, followed by a post hoc comparison according to Conover (Bortz et al. 2008). Significant differences were determined at the 0.05 levels of confidence.

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Table 1. Oligonucleotide primers used in qPCR

GenBank accession number	Gene and primer name		Sequence 5′–3′[a]	PCR product length
X69885	*Actin* (*Act*)	Fw	CGGAATCCACGAGACTACATAC	230 bp
		Rev	GGGAAGCCAAGATAGAGC	
AJ429078	*Tobacco mosaic virus coat protein* (*TMV CP*)	Fw	CTTGTCATCAGCGTGGGC	165 bp
		Rev	AAGTCACTGTCAGGGAAC	
U62546	*Tobacco necrosis virus coat protein* (*TNV CP2*)	Fw	CTTCTGGGCTTAGTTTCC	351 bp
		Rev	CCTGCGTTCTTGTCGTA	
U49241	*Pathogenesis relat ed-1* (*PR-1*)	Fw	ACTTGGGACGACGAGGTA	197 bp
		Rev	GCACAATGATTTGAGCC	
AF190634	*Salicylic acid glucosyltransferase* (*SAGT*)	Fw	AAAGAAGTTGGCTCGGATA	514 bp
		Rev	TTGGCTTGAAGACACTAAGG	
AY206006	*Glutathione S-transferase Tau1* (*GSTTau1*)	Fw	GATGGCAGAAGTGAAGTTG	487 bp
		Rev	CTCCTAGCCAAAATSCCA	
AY206005	*Glutathione S-transferase Phi* (*GSTPhi*)	Fw	CTGGKGAWCACAAGAAGC	490 bp
		Rev	GCCAARATATCAGCACACC	
M60055	*Salicylate hydroxylase* (*NahG2*)	Fw	ACGCCCTAGTAACTCACCTC	184 bp
		Rev	CCCTGACCTTCCAGCACAT	

[a] Degenerate bases are coded as follows: K = G or T; R = A or G; W = A or T.

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