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Differential roles of salicylic acid and glutathione in resistance of tobacco to tobacco mosaic virus and tobacco necrosis virus

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RESEARCH ARTICLE

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ABSTRACT

It is well-established that salicylic acid (SA) and glutathione are key components of plant defense against viruses. Our earlier research showed that elevated glutathione in tobacco correlated with increased SA levels resulting in enhanced resistance to Tobacco mosaic virus (TMV). We also demonstrated that glutathione could maintain TMV resistance in SA-deficient transgenic tobacco (Künstler et al., 2019). In the present study, we tested how SA and glutathione affect plant defense to Tobacco necrosis virus (TNV). Our results show that glutathione overproduction in tobacco had no effect on TNV levels and elevated glutathione in SA deficient tobacco could not compensate for the lack of SA as opposed to TMV infections. An analysis of SA and glutathione levels was conducted in virus-inoculated plants. An increase in SA levels was a characteristic of TMV infection, but not of TNV, while glutathione levels increased in response to both viruses. However, SA is necessary for maintaining plant defense to both TMV and TNV because the absence of SA leads to

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increased susceptibility to both viruses. In overall high levels of glutathione seem to be clearly associated with a higher degree of resistance to TMV but not to TNV. This study may contribute to a better understanding of plant defense responses against viruses.

KEYWORDS

glutathione, plant disease resistance, salicylic acid, tobacco mosaic virus, tobacco necrosis virus

INTRODUCTION

Over the past few decades, the role of various phytohormones in activating plant defense responses to pathogens has come into the spotlight (Shafqat et al., 2024). Salicylic acid (SA, 2-hydroxybenzoic acid) has been identified as a key component in plants' defense against biotrophic pathogens, including viruses (Lee et al., 2016; Murphy et al., 2020; Incarbone et al., 2023; Király et al., 2024). SA coordinates the plant's defense against viruses by participating in the perception of virus-derived signals, inhibiting virus replication and local (cell-to-cell) or systemic movement (Zhao and Li, 2021). In the absence of SA, plant defenses against viruses are severely compromised. The NahG tobacco line (*Nicotiana tabacum* cv. Xanthi NN NahG) expresses a bacterial *nahG* gene that encodes a salicylate hydroxylase enzyme, which transforms SA into catechol. Consequently, this transgenic line exhibits reduced SA accumulation and disease resistance signaling, making it more susceptible to e.g. Tobacco mosaic virus (TMV) and Tobacco necrosis virus (TNV) infection as compared to wild-type plants (Delaney et al., 1994; Künstler et al., 2019; Garcia et al., 2023).

When resistant tobacco plants are infected by viruses, they quickly produce reactive oxygen species (ROS) which leads to oxidative stress (Hernández et al., 2016). It is not surprising that certain viruses are able to suppress the formation of ROS and SA, allowing the virus to replicate and spread in infected cells (Zvereva et al., 2016). The accumulation of ROS in infected cells affects the redox state, activating thereby plant defense and helping the plants to adapt to viral infections (Xu et al., 2024). In fact, when there is an excess of ROS accumulation plant cells activate various enzymatic and non-enzymatic antioxidants to prevent ROS induced damage. Glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) is one of the most abundant non-enzymatic antioxidants in plant cells that plays a principal role in the elimination of ROS via the ascorbate-glutathione cycle (Foyer and Kunert 2024). Moreover, GSH and its oxidized disulfide form GSSG compose a redox buffer that contributes to the maintenance of cellular redox homeostasis. The activation of ROS and antioxidant responses in infected plant cells affect various biochemical processes associated with plant defense reactions to different pathogens (Zhu et al., 2021; Noctor et al., 2024). Interestingly, ROS signaling affects SA-mediated signaling, and vice versa (Myers et al., 2023). Overall, the interplay between these three key components (ROS, SA, GSH) fundamentally determines the outcome of the virus-tobacco interaction.

It is known that in SA-deficient NahG tobacco glutathione levels are significantly lower than in wild-type Xanthi plant (Király et al., 2002). Furthermore, exogenous SA-treatments of Xanthi tobacco result in increased GSH levels along with an enhanced resistance to TMV (Fodor et al., 1997). Previously we have attempted to gain a deeper insight into the interaction of GSH and SA during defense responses to TMV infection in tobacco plants (Künstler et al., 2019). Our results

demonstrated that GSH overproduction in transgenic tobacco (*N. tabacum* cv. Burley TRI-2 and CEMK-9) resulted in milder symptoms and reduced TMV levels, as compared to wild type plants (*N. tabacum* cv. Burley). Moreover, SA deficiency in NahG tobacco (*N. tabacum* cv. Xanthi NN NahG) led to enhanced TMV symptoms and elevated virus levels, as compared to control plants (*N. tabacum* cv. Xanthi NN). We demonstrated that in SA-deficient NahG tobacco the elevation of in planta GSH levels by crossing with glutathione overproducing plants (*N. tabacum* cv. Burley TRI-2 and CEMK-9) confers enhanced resistance to TMV manifested as both reduced symptoms (i.e. suppression of hypersensitive-type localized necrosis) and lower virus titers. This suggests that elevated levels of GSH in TMV-infected tobacco can compensate for SA deficiency to maintain virus resistance (Künstler et al., 2019). The question arises how elevated GSH levels affect other plant viruses. In the present study, we tested how high levels of GSH affect plant defense to Tobacco necrosis virus (TNV). Moreover, we also tested whether glutathione can improve suppressed plant defenses in the absence of SA. This information could help us to better understand the mechanisms of plant defense against viruses.

MATERIAL AND METHODS

Plant materials

The following tobacco cultivars and lines were utilized in the experimental procedures:

- *N. tabacum* L. cv. Samsun nn.
- *N. tabacum* cv. Xanthi NN wild type and its SA-deficient line *N. tabacum* cv. Xanthi NN NahG expressing the *nahG* transgene encoding salicylate hydroxylase (Gaffney et al., 1993).
- *N. tabacum* cv. Burley NN wild type and its two glutathione overproducer transgenic lines *N. tabacum* cv. Burley NN CEMK-9, and *N. tabacum* cv. Burley NN TRI-2. The CEMK-9 transgenic tobacco line overexpresses two different transgenes encoding serine acetyltransferase (*EcSAT*) and O-acetylserine (thiol)-lyase (*EcOASTL*) while the TRI-2 line overexpresses three transgenes encoding serine acetyltransferase (*EcSAT*), γ-glutamylcysteine synthase (*EcGSH1*) and phytochelatin synthase (*SpPCS*) which are involved in cysteine and glutathione biosynthesis (Liszewska et al., 2001; Wawrzyński et al., 2006). *N. tabacum* cv. Burley and its transgenic lines are a kind gift of Dr. Agnieszka Sirko (Institute of Biochemistry and Biophysics, Polish Academy of Sciences Warsaw, Poland).
- To increase the glutathione content of SA-deficient tobacco, we crossed the SA-deficient NahG tobaccos with glutathione overproducer tobacco lines (CEMK-9, TRI-2) by mechanical pollination (hand pollination) and using the F_1 generation individuals for further experiments. To prove the success of crossings the presence of transgenes (*nahG*, *SAT*, *OASTL*, *GSH1*, and *PCS*) originating from different parents was verified in F_1 generation individuals by polymerase chain reaction (PCR) with primers described earlier (*PpnahG*, *EcSAT*, *EcOASTL*, *EcGSH1* and *SpPCS*) (Gaffney et al., 1993; Blaszczyk et al., 1999; Liszewska et al., 2001; Wawrzyński et al., 2006). First, we used glutathione overproducers (CEMK-9 and TRI-2) as male (♂) and SA-deficient NahG plants as female (♀) parents. Reciprocal crossings were also performed and the resulting F_1 plants gave similar experimental results.

Tobacco plants were grown in the greenhouse with standard parameters (temperatures between 20 and 23 °C, approximately 16 h of daylight with daily watering).

Virus inoculation

A Hungarian TNV isolate serologically related to the E strain of TNV-D (Pogány et al., 2004) was propagated on tobacco (*N. tabacum* cv. Samsun nn). TNV-inoculated leaves displaying local necrotic lesions were used to mechanically inoculate plants. TNV inoculation was performed as we described earlier in case of TMV (Künstler et al., 2019). In brief, TNV infected leaves homogenized in tap water (1 g of leaf in 10 mL of water) with silicon carbide (carborundum) powder as an abrasive were used to infect third and fourth true leaves counted from below (the total number of mature leaves was between six and eight) of 60–70 day-old tobacco by mechanical inoculation. Mock inoculations with tap water and carborundum powder were also performed as controls. TNV induced symptoms were visually evaluated 5 days after virus inoculations (DAI) when local necrotic lesions are fully developed.

Monitoring TNV accumulation

To analyze TNV accumulation, inoculated leaves were collected 5 DAI and ground in liquid nitrogen. A total of 200 mg of fresh leaf tissue was collected per sample. Each sample represents six leaves from three different plants. Total RNA (plant and virus) contents were isolated by the Plant Total RNA Extraction Miniprep System Kit (Viogene Biotek Corp., Taiwan) as outlined in the kit's instructions. After RNA isolation, DNase treatment with RQ1 RNase-Free DNase was performed (Promega Corp., WI, U.S.A.). For reverse transcription (RT), a total of 1 µg of RNA was used for each sample. RT was done with a RevertAid™ H^- cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania) according to manufacturer's instructions using both TNV coat protein gene (*TNVcp*) reverse primer (*TNV-CP2 rev*) and an oligo-dT primer. In the next step a quantitative real-time PCR (qPCR) assay was conducted to determine the relative expression of *TNV-CP2* using 2.5 µL of 20-fold diluted cDNA in a total reaction volume of 15 µL (Künstler et al., 2019) with the SYBR FAST Readymix reagent (KAPA Biosystems Ltd., South Africa). PCR amplifications were carried out in a CFX-96 real-time thermocycler (Bio-Rad Corp., CA, U.S.A.) by using a standard program: 95 °C for 2 min, 40 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. Gene expression was normalized to a tobacco *actin* (*NtAct*) gene as a reference (Király et al., 2024). Relative gene expression was calculated by employing the comparative C_T ($2^{-\Delta\Delta CT}$) method (Schmittgen and Livak, 2008). Expression levels of *TNVcp* in all samples are related to that in *N. tabacum* cv. Burley (arbitrary unit 1). Oligonucleotide primers used in these experiments are: tobacco actin gene (*NtAct*, GenBank accession X69885) 5′-CGGAATCCACGAGACTACATA-3′ (5′ primer) and 5′-GGGAAGCCAAGATAGAGC-3′ (3′ primer); Tobacco necrosis virus coat protein gene (*TNV CP2*, GenBank accession U62546) 5′-CTTCTGGGCTTAGTTTCC-3′ (5′ primer) and 5′-CCTGCGTTCTTGTCGTA-3′ (3′ primer) (Király et al., 2024). Primers were designed by us with the aid of the Primer Premier 5 program (PREMIER Biosoft International, CA, U.S.A.).

Determination of free and bound SA

Free and bound (conjugated) forms of SA were detected by high performance liquid chromatography (HPLC) analysis by using ortho-anisic acid as an internal standard (Meuwly and Metraux 1993) with small modifications. To analyze SA levels 1 g of tobacco from inoculated leaves were sampled 5 DAI and ground in quartz sand and liquid nitrogen. Each sample represents six leaves from three different plants. The ground tissue was extracted with 70%

methanol. Bound SA was hydrolyzed with 4 M hydrochloric acid. The following steps of the extraction and separation were carried out as described in detail previously (Cole et al., 2004; Pál et al., 2005). SA was quantified fluorimetrically with W474 scanning fluorescence detector (Waters Corp., MA, U.S.A.), with excitation at 305 nm and emission at 407 nm.

Determination of reduced (GSH) and oxidized (GSSG) glutathione

Plant tissue extraction and analyses of GSH and GSSG were carried out as described previously (Rellán-Álvarez et al., 2006; Künstler et al., 2019). Leaf samples (500 mg) were collected from inoculated leaves 5 DAI. Each sample represents six leaves from three different plants. Collected leaves were ground in liquid nitrogen and the tissue powder was suspended in four volumes of cold (4 °C) extraction solution which contains 5% (w/v) stabilized metaphosporic acid (Acros Organics, USA) and 1 mM EDTA in 0.1% formic acid, supplemented with 1% (w/v) polyvinyl-polypyrrolidone just before use. The suspension was centrifuged at 14,000*g* for 15 min at 4 °C. The supernatant was collected and the pellet was resuspended with two volumes of the same cold extraction solution and centrifuged again. Both supernatants were combined and filtered through 0.45 µm Cromafil polyamide membrane filters (Macheray-Nagel, Germany). After HPLC separation and electrospray ionization mass spectrometric analyses (HPLC-ESI/MS) were performed with an HPLC MS system (Shimadzu Corp., Japan). Samples were analyzed in negative ion mode. GSH was detected at $m/z = 306$, GSSG at $m/z = 611$. Unlabeled, analytical grade GSH and GSSG (Sigma-Aldrich, USA) were used as external standards for quantitation of GSH and GSSG levels in tobacco leaves. The GSH/GSSG ratio was calculated as previously described (Foyer et al., 1991).

Statistical analysis

All analyses were performed using three independent biological experiments with three technical replicates per biological sample. Statistical evaluations were conducted using the Statistica 13 software (TIBCO Software, CA, U.S.A.). The normality of data distribution was assessed using the Shapiro–Wilk test and homogeneity of variances by Levene's test. ANOVA and Tukey HSD tests were employed, and differences at $P \leq 0.05$ considered statistically significant.

RESULTS

Our aim was to gain a deeper insight into the interaction of salicylic acid (SA) and glutathione during defense responses to Tobacco necrosis virus (TNV) infection in tobacco, as compared to our earlier results concerning Tobacco mosaic virus (TMV) (Künstler et al., 2019). First, we tested the individual effects of elevated glutathione levels or SA deficiency on TNV infection. *N. tabacum* cv. Burley and its glutathione overproducer transgenic lines (*N. tabacum* cv. Burley CEMK-9 and TRI- 2), as well as *N. tabacum* cv. Xanthi and SA deficient *N. tabacum* cv. Xanthi NahG lines were used. Tobacco lines were inoculated with TMV and TNV and disease symptoms were evaluated 5 DAI when necrotic lesions are fully developed. According to our previous research, elevated glutathione levels contribute to the resistance of tobacco to TMV, manifested as milder necrotic symptoms in glutathione overproducer lines (TRI-2, CEMK-9) as compared to the wild type Burley cultivar (Künstler et al., 2019) (Fig. 1). In contrast, no difference was detectable in TNV symptoms between glutathione-overproducer and wild type plants (Fig. 1). SA deficiency significantly impaired virus resistance, both TMV and TNV causing more severe necrotic symptoms

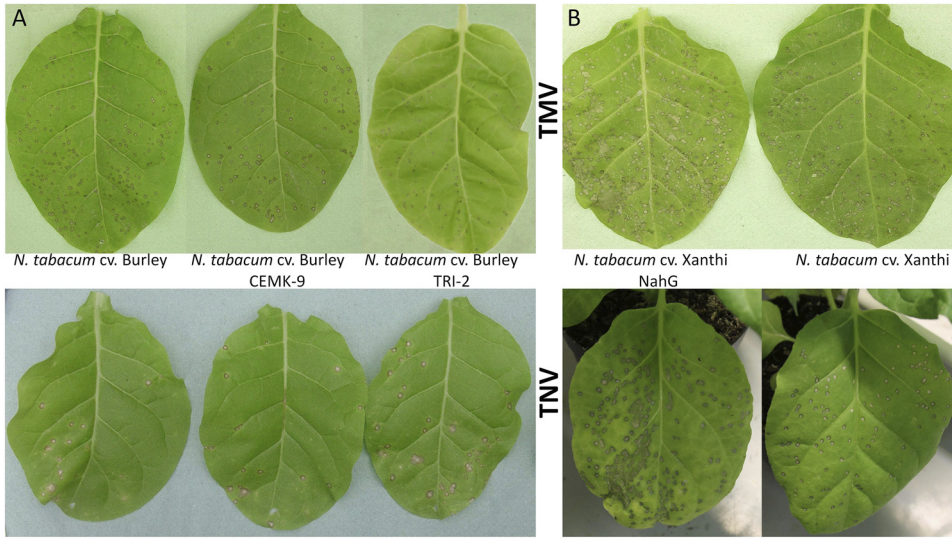

Fig. 1. The effect of glutathione overproduction (A) or salicylic acid deficiency (B) on symptoms caused by Tobacco mosaic virus (TMV) and Tobacco necrosis virus (TNV) in tobacco leaves, five days after inoculation. *Nicotiana tabacum* cv. Burley wild type and its glutathione overproducer lines (CEMK-9 and TRI-2) and salicylic acid deficient *N. tabacum* cv. Xanthi NahG and its wild type control, *N. tabacum* cv. Xanthi

in SA-deficient NahG tobacco, as compared to wild type Xanthi plants (Fig. 1). To assess the role of GSH in SA-mediated defense we crossed the SA-deficient NahG line with GSH overproducer tobacco (TRI-2, CEMK-9) and used their F_1 hybrids. Our results showed that inoculating the TRI-2 x NahG F_1 and CEMK-9 x NahG F_1 hybrids with TNV resulted in severe symptoms similar to those of the NahG parent (Fig. 2). However, the symptoms of TMV infection in the F_1 hybrids are milder than in the NahG parent, in fact, corresponding to those in wild type plants (Figs 1 and 3). Thus, high glutathione levels contribute to reducing severe symptoms in the absence of salicylic acid in response to TMV (Künstler et al., 2019) but not TNV.

In addition to evaluating the necrotic symptoms induced by the viruses, we monitored the relative levels of TMV and TNV by quantifying expression of the genes that encode the coat proteins of TMV and TNV (*TMVcp* and *TNVcp*) in inoculated tobacco 5 DAI using quantitative reverse transcription real-time PCR. The results of the symptom assessment were consistent with changes in viral levels. Glutathione overproduction resulted in reduced TMV levels in TRI-2 and CEMK-9 lines as compared to wild type Burley; however, glutathione overproduction had no effect on TNV levels (Fig. 4). SA deficiency in the cv. Xanthi NahG line resulted in elevated TMV and TNV levels, as compared to the control cv. Xanthi (Fig. 4). In the F_1 hybrids, TMV levels were intermediate between the glutathione overproducer (CEMK-9, TRI-2) and SA deficient NahG parents (see also Künstler et al., 2019), while TNV levels in F_1 hybrids were similar to those in the NahG parent (Fig. 4).

Additionally, an analysis of free and bound SA levels was conducted in uninoculated, mock inoculated, TMV or TNV inoculated plants 5 DAI. The levels of free SA were elevated in glutathione overproducer lines (CEMK-9, TRI-2), as compared to wild type Burley in

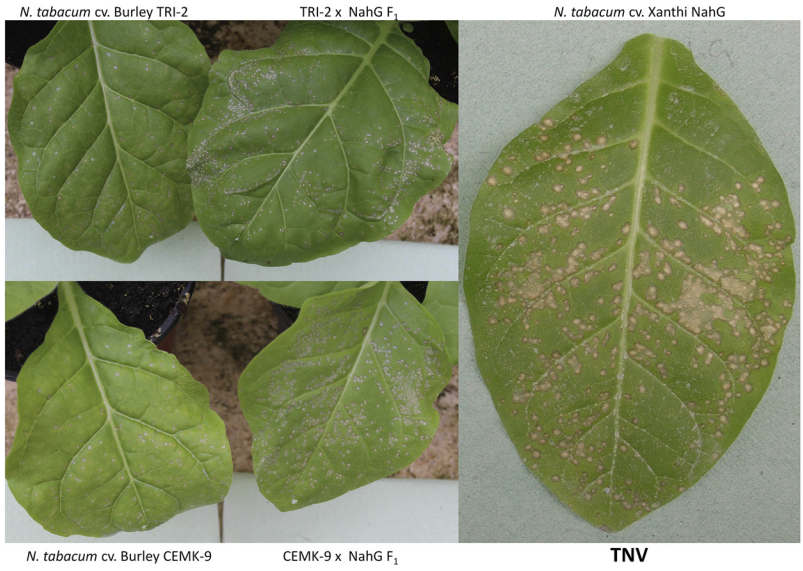

Fig. 2. Disease symptoms caused by Tobacco necrosis virus (TNV) five days after inoculation in glutathione overproducer *Nicotiana tabacum* cv. Burley TRI-2, CEMK-9, and salicylic acid deficient *N. tabacum* cv. Xanthi NahG, as well as their F_1 hybrids (TRI-2 X NahG, CEMK-9 X NahG)

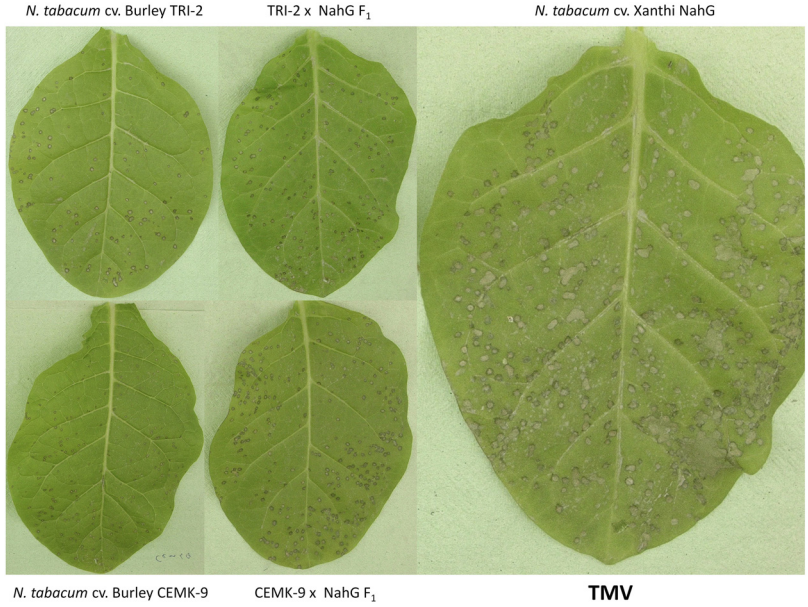

Fig. 3. Disease symptoms caused by Tobacco mosaic virus (TMV) four days after inoculation in glutathione overproducer *Nicotiana tabacum* cv. Burley TRI-2, CEMK-9, and salicylic acid deficient *N. tabacum* cv. Xanthi NahG, as well as their F_1 hybrids (TRI-2 X NahG, CEMK-9 X NahG)

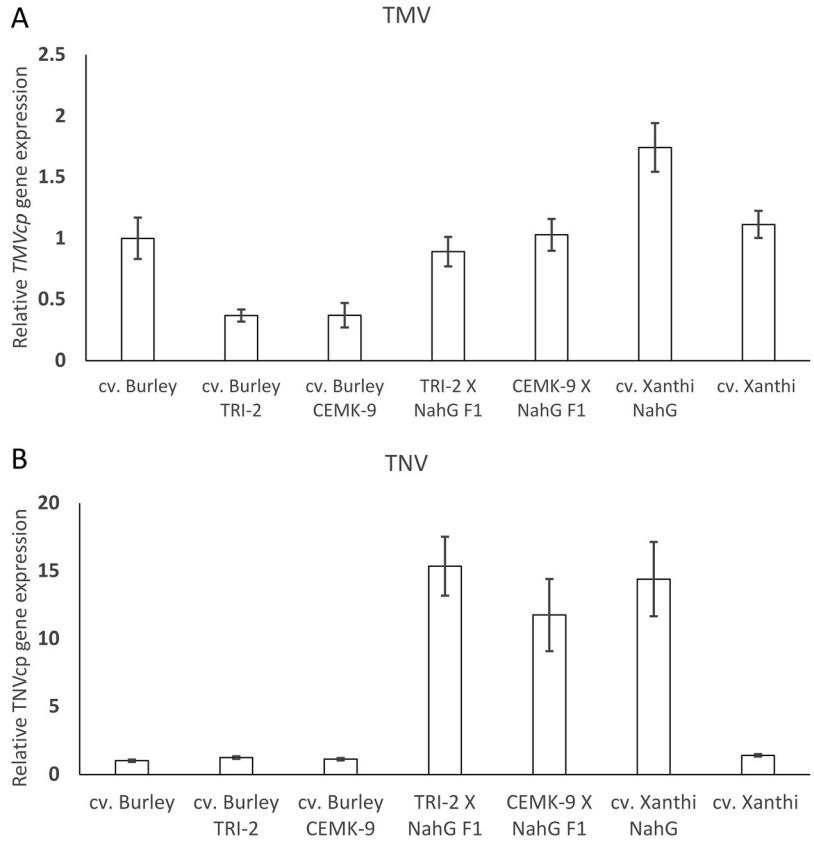

Fig. 4. Relative levels of Tobacco mosaic virus (TMV) (A) and Tobacco necrosis virus (TNV) (B) five days after inoculation in tobacco lines (*Nicotiana tabacum* cv. Burley and its glutathione overproducer lines *N. tabacum* cv. Burley TRI-2, CEMK-9, salicylic acid deficient *N. tabacum* cv. Xanthi NahG and wild type *N. tabacum* cv. Xanthi). Quantification of virus levels was achieved by using quantitative reverse transcription real-time PCR of the TMV and TNV coat protein-encoding genes (*TMVcp* and *TNVcp*) in inoculated tobacco lines 4 days after inoculation. As an internal control, a tobacco *Actin* gene was used. Expression levels of *TMVcp* and *TNVcp* in all samples are related to that in tobacco cv. Burley (arbitrary unit 1). Vertical bars show the average of three independent experiments. Error bars represent standard deviation. Values with different letters above bar graphs are significantly different at $P \leq 0.05$

uninoculated plants (Fig. 5). As expected, low levels of SA were measured in the plants expressing the *nahG* transgene. TMV inoculation increased the levels of free SA, especially in the glutathione overproducer lines, as compared to the wild-type Burley plants. However, TNV had no effect on SA levels (Fig. 5). Interestingly, TMV inoculation, as opposed to TNV, elevated free SA contents even in SA deficient plants (i.e. in NahG and TRI-2 x NahG F_1 and CEMK-9 x NahG F_1 hybrids) (Fig. 5). The changes in bound salicylic acid levels were similar to those observed in free salicylic acid (Fig. 5). In uninoculated F_1 hybrids (CEMK-9 x NahG and TRI-2 x NahG) levels of both free and bound SA were as low as in the NahG parent. On the other hand, TMV inoculation, as opposed to TNV, elevated free SA contents in these F1 hybrids (Fig. 5).

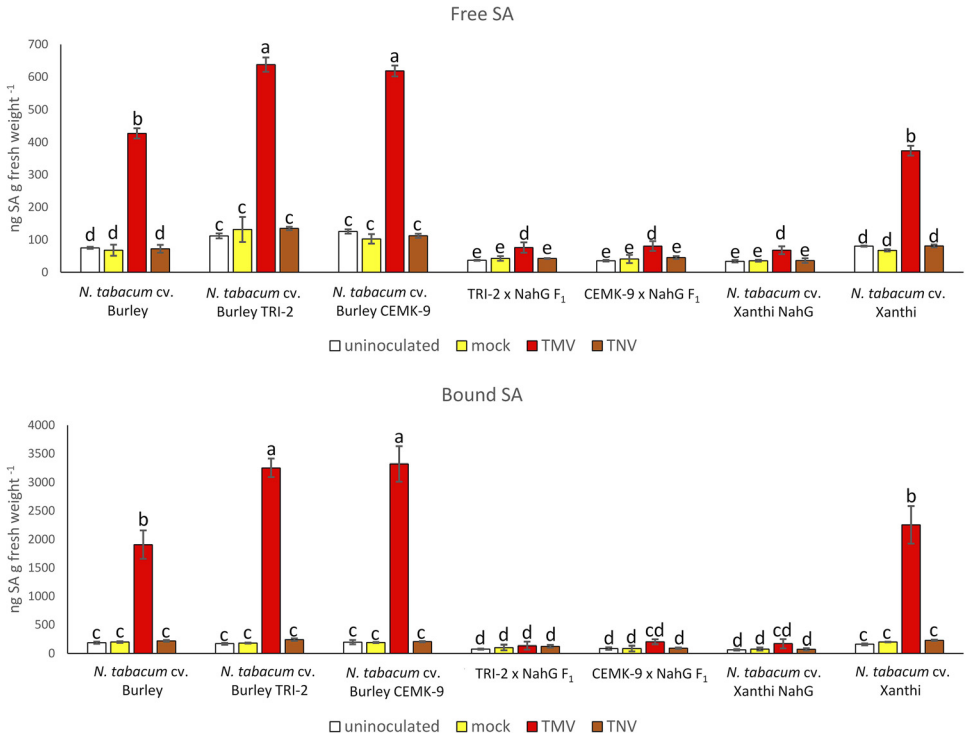

Fig. 5. Levels of free and bound salicylic acid (SA) in uninoculated, mock-inoculated, Tobacco mosaic virus (TMV) and Tobacco necrosis virus (TNV) inoculated *Nicotiana tabacum* cultivars/lines. Detection of SA was performed 5 days after inoculation. For the description of tobacco lines see the legend of Fig. 4. Vertical bars show the average of three independent experiments. Error bars represent standard deviation. Values with different letters above bar graphs are significantly different at $P \leq 0.05$

Overall, an increase in SA levels was a characteristic of TMV infection. However, SA levels did not change as a result of TNV infection. Nevertheless, SA is necessary for maintaining plant defense to both TMV and TNV, since the absence of SA in NahG plants leads to increased susceptibility to both viruses (Figs. 1 and 4).

We have also assessed levels of reduced (GSH) and oxidized (GSSG) glutathione in uninoculated, control inoculated (mock), TNV and TMV-inoculated leaves of these tobaccos, 5 DAI. Glutathione overproducer lines had higher glutathione levels compared to wild-type plants even without infection. Interestingly, glutathione levels were significantly reduced in SA deficient NahG plants (but not in SA-deficient TRI-2 x NahG and CEMK-9 x NahG F_1 hybrids) as compared to all the other lines. TMV inoculation further increased reduced and oxidized glutathione levels in CEMK-9 and TRI-2 glutathione overproducer lines, but TNV infection did not affect glutathione levels (Fig. 6A). High levels of glutathione seem to be clearly associated with a higher degree of resistance to TMV but not to TNV in TRI-2 and CEMK-9 plants. On the other hand, the low levels of glutathione in NahG plants (but not in SA-deficient TRI-2 x NahG and CEMK-9 x NahG F_1 hybrids) did not change in response to virus (TMV and TNV) inoculations. Interestingly, in uninoculated F_1 tobacco hybrids (CEMK-9 x NahG and TRI-2

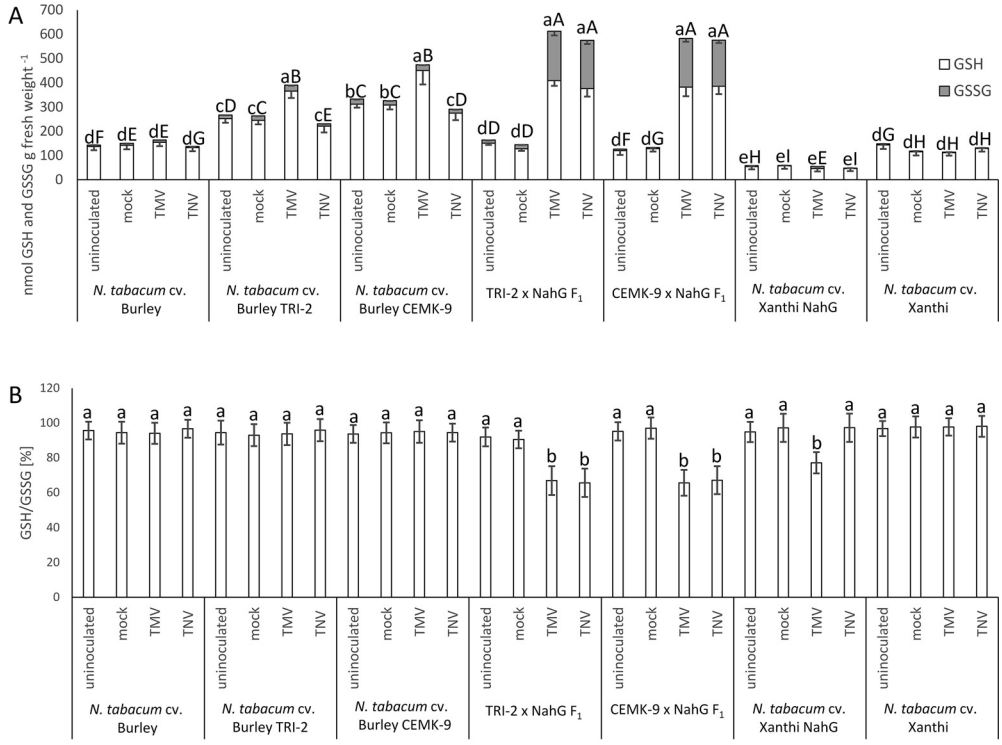

Fig. 6. Reduced (GSH) and oxidized (GSSG) glutathione levels (A) and GSH/GSSG ratios (B) in uninoculated, mock-inoculated, Tobacco mosaic virus (TMV) and Tobacco necrosis virus (TNV) inoculated *Nicotiana tabacum* cultivars/lines. Detection of glutathione was performed 5 days after inoculation. For the description of tobacco lines see the legend of Fig. 4. Vertical bars show the average of three independent experiments. Error bars represent standard deviation. Values with different letters above bar graphs are significantly different at $P \leq 0.05$. In Fig. 6A the significance of GSH is denoted by lowercase letters, while capital letters refer to GSSG

x NahG) glutathione levels were intermediate between those of the parents and corresponding to glutathione levels in wild type tobacco. However glutathione levels were extraordinarily high in TMV and TNV-inoculated F_1 hybrids (CEMK-9 x NahG and TRI-2 x NahG), primarily due to a several fold increase in levels of GSSG, as compared to controls (Fig. 6A). The GSH/GSSG ratio as a marker of oxidative stress in plant cells was also calculated. The several fold increase in levels of GSSG in TMV and TNV inoculated F_1 hybrids (CEMK-9 x NahG and TRI-2 x NahG) and TMV inoculated NahG plants led to a redox imbalance as compared to all the other lines indicating immense oxidative stress (Fig. 6B).

DISCUSSION

Numerous studies have shown that altering glutathione levels in plants can significantly affect their ability to resist viruses. Our previous research showed that artificial elevation of GSH contribute to

plant defense of tobacco against TMV characterized by milder symptoms and lower virus levels (Künstler et al., 2019). Pretreating tobacco leaves with the synthetic cysteine precursor (cysteine is a sulfur containing amino acid required for glutathione biosynthesis) R-2-oxo-4-thiazolidine-carboxylic acid (OTC) prior to virus inoculation increased GSH contents and significantly reduced TMV coat protein levels (Gullner et al., 1999). Moreover, OTC treatments also reduced Zucchini yellow mosaic virus accumulation in infected pumpkin (Zechmann et al., 2007). Silencing the genes that encode enzymes crucial for GSH biosynthesis severely compromised TMV and Potato virus X (PVX) resistance of *N. benthamiana* (De et al., 2018; Zhu et al., 2021). However, in Plum pox virus-inoculated peach, OTC treatments increased GSH contents but did not significantly reduced virus levels (Clemente-Moreno et al., 2012). Our present study also revealed that glutathione overproduction in transgenic tobacco plants (*N. tabacum* cv. Burley CEMK-9 and TRI-2) did not enhance plant defense against TNV. Interestingly, TMV infection increased the already high glutathione levels in the transgenic plants (CEMK-9 and TRI-2) that overproduce glutathione 5 DAI. In contrast, TNV infection has no effect on glutathione levels in these plants. The examples listed above demonstrate that GSH accumulation in plant-virus interactions is not always beneficial. In certain cases, an increase in glutathione levels does not necessarily correspond with enhanced virus resistance, when GSH cannot efficiently enhance certain elements of the plant defense response to virus infections (Clemente-Moreno et al., 2012; the present study).

One way how GSH could enhance plant defense against viruses is that it activates the SA-mediated signaling pathway (Zhu et al., 2021). It has been demonstrated that in tobacco, TMV infection increases the expression of *γ-glutamylcysteine synthetase* and *glutathione synthetase* genes which resulted in the elevation of GSH levels promoting SA accumulation and downstream signaling processes culminating in enhanced resistance to TMV (Zhu et al., 2021). Indeed, the artificial elevation of GSH contents in plants led to markedly increased SA levels as well as up-regulation of expression of the SA-mediated resistance-associated gene *pathogenesis related-1* (*PR-1*) and enhanced resistance to viral, bacterial and fungal pathogens (Ghanta et al., 2011, 2014; Künstler et al., 2019). The activation of SA-mediated signaling coordinates the plant's defense against viruses by inhibiting virus replication and spread (Zhao and Li, 2021). SA induces plant defense through conformational changes of non-expressor of pathogenesis-related 1 protein (NPR1) which acts as a master regulator of SA-mediated defense (Mou et al., 2003; Chen et al., 2021). In an uninduced state NPR1 is present as an oligomer in the cytoplasm held together by disulfide bonds. During infection, SA induces the oligomer-monomer switch (activation) of NPR1, which is catalyzed by thioredoxins (TRXs). This process uses low-molecular-weight (LMW) thiols, mostly glutathione, as a substrate (Tada et al., 2008). Interestingly, increased expression of a thioredoxin gene (*NtTRXh*) was detected in glutathione overproducing tobacco (Ghanta et al., 2011). Activated NPR1 monomers translocate into the nucleus and activate the expression of pathogenesis related genes which may participate in plant antiviral defense (Park et al., 2004; Šindelářová and Šindelář, 2005). Our earlier results suggest that artificial elevation of glutathione enhanced plant defense to TMV likely through the NPR1-dependent salicylic acid-mediated pathway. Glutathione overproducer tobacco lines showed elevated SA levels and enhanced plant defense to TMV (Künstler et al., 2019). Interestingly, artificial elevation of glutathione was able to compensate the suppressed plant defense to TMV in transgenic NahG tobacco where the amount of SA is very limited (Künstler et al., 2019). Based on the above information and our results, we have developed a theoretical model explaining how the interplay between SA and glutathione contributes to plant defense against TMV

(Fig. 7). In wild-type *N. tabacum* cv. Xanthi NN and cv. Burley NN tobacco plants, which are resistant to TMV, virus infection activates the salicylic acid (SA)-mediated pathway through an NPR1 oligomer-monomer switch catalyzed by thioredoxin enzymes, resulting in resistance to TMV. SA and GSH are required for the proper functioning of thioredoxins (Tada et al., 2008). In *N. tabacum* cv. Xanthi NahG plants, low SA and glutathione levels presumably do not favor the thioredoxin catalyzed NPR1 oligomer-monomer switch, resulting in impaired plant defense to TMV. The F_1 generation of NahG *x* CEMK-9 and NahG *x* TRI-2 hybrids accumulate low levels of SA, which is partially compensated by increased GSH levels – probably activating NPR1 monomer production – resulting in a restored plant defense against TMV. In glutathione overproducer lines (TRI-2 and CEMK-9) elevated glutathione and SA levels enhance plant defense to TMV (likely

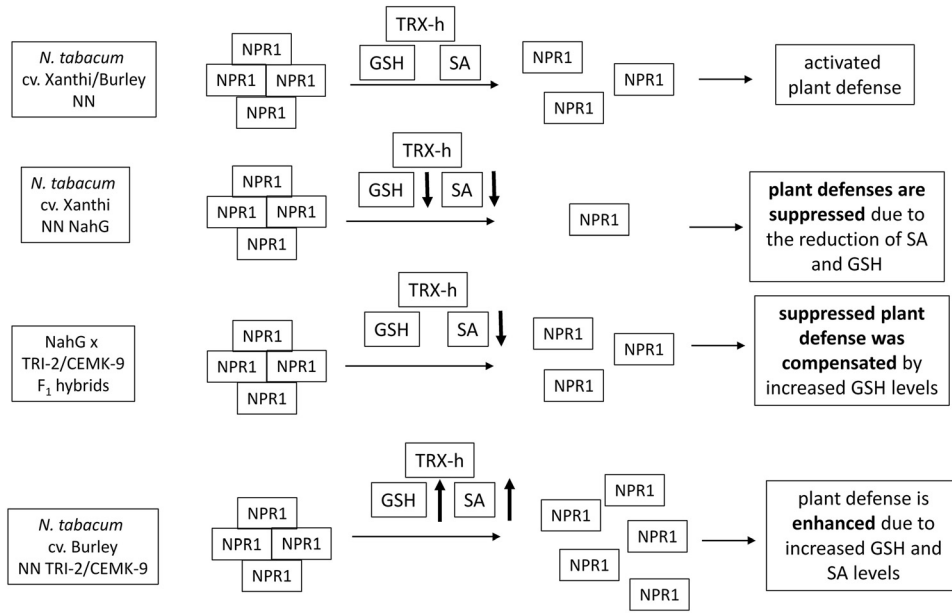

Fig. 7. A theoretical model explaining how the interplay between salicylic acid (SA) and glutathione (GSH) contributes to plant defense against TMV. In wild-type (wt), Tobacco mosaic virus (TMV) resistant *Nicotiana tabacum* cv. Xanthi NN and cv. Burley NN tobaccos, virus infection activates the SA-mediated pathway through an oligomer-monomer switch in the defense regulator non-expressor of pathogenesis-related 1 protein (NPR1) catalyzed by thioredoxin (TRX-h) enzymes and resulting in resistance to TMV. SA and GSH are required for the proper functioning of thioredoxins (Tada et al., 2008). In *N. tabacum* cv. Xanthi NahG plants, low SA and GSH levels presumably do not favor the thioredoxin catalyzed NPR1 oligomer-monomer switch causing impaired plant defense to TMV. NahG x CEMK-9 and NahG x TRI-2 F_1 tobacco hybrids also produce low amounts of SA, which is partially compensated by an increase in GSH to wild type levels (Künstler et al., 2019). This probably activates NPR1 monomer production resulting in a restored plant defense to TMV. In GSH overproducer tobaccos (TRI-2 and CEMK-9) elevated GSH and SA levels enhance plant defense to TMV – likely through NPR1 – resulting in reduced TMV levels as compared to wild type plants. Arrows pointing downwards represent lower than wild type levels of GSH and SA, while upward pointing arrows depict higher than wild type SA and GSH. No arrows = wild type SA and GSH levels

through NPR1) resulting in reduced TMV levels as compared to wild type plants (Fig. 7). In contrast, elevated glutathione and SA levels have no effect on the TNV resistance of these plants, however, SA is necessary for plant defense against TNV, since the low SA levels in NahG tobacco and their F_1 hybrids resulted in elevation of TNV titers. Recently, others have also presented that SA is required for plant defense to TNV in tobacco (Garcia et al., 2023).

In comparing the two viral infections (TMV and TNV) in tobacco, we found significant differences in plant defense processes. Elevation of GSH levels in tobacco resulted in enhanced plant resistance to TMV but not to TNV. Since a decrease in the amount of SA in tobacco was associated with an increase in the levels of both viruses, it seems that SA is necessary for the proper plant resistance to both of these viruses. Importantly, however, the decrease in resistance caused by salicylic acid deficiency could be offset by raising glutathione levels to combat TMV, although it had no effect on TNV infections. In our opinion, salicylic acid and glutathione may modulate the NPR1 protein, exerting thereby differential effects on plant defense to TMV vs. TNV. Recent studies have shown that certain viruses are able to suppress SA-mediated plant defense by impairing NPR1 functionality through inducing its degradation, altering its translocation, or inhibiting its post-translational modification (Liu et al., 2023; Jiang et al., 2025). However, it is unclear whether NPR1 suppression is a general phenomenon among viruses. Nevertheless, further studies are needed to elucidate how GSH and SA modulate NPR1 and participate in plants resistance to TMV and TNV.

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