NATIONAL RESEARCH, DEVELOPMENT AND INNOVATION OFFICE PD108455 FINAL REPORT
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Title of the completed project:

Role of glutathione in salicylic acid-mediated plant disease resistance to viral and fungal infections.

Scientific background of the research topic

Plants produce several different hormones like salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), etc. which play a pivotal role in plant resistance to biotic stress (pathogenic infections). Among these plant hormones, SA is an important determinant of plant defense, since its production is actively induced primarily during resistance to infections caused by biotrophic pathogens (Bary and Jones, 2009). On the other hand, SA-deficient mutant/transgenic plants display enhanced susceptibility to these pathogens. SA accumulation during pathogen attack triggers the activation of defense genes (e.g. pathogenesis-related /PR/ genes) which can then induce a so-called systemic acquired resistance (SAR) to the same or other pathogens (Ward et al., 1991; Gaffney et al., 1993). Glutathione (GSH) plays an important role as an antioxidant, and is also known as a regulator of plant signaling during resistance to pathogen attack (Klapheck et al. 1992; Vanacker et al., 1998). Moreover, previous papers have shown that GSH has a significant impact on SA-mediated plant resistance signaling to both hemibiotrophic and necrotrophic pathogens (Han et al., 2012; Ghanta et al., 2011 a, b; Kovacs et al., 2015). However, the relation of GSH and SA during resistance responses to biotrophic pathogens is currently not known. Therefore, **the main goal of this proposal was to investigate the role of GSH in SA-mediated plant resistance to biotrophic pathogens (obligate parasites), e.g. powdery mildews and viruses.**

Results

Effects of *in planta* enhancement of GSH biosynthesis on resistance to *Tobacco mosaic virus* in the F_1 generation of a cross between SA-deficient and GSH overproducer *Nicotiana tabacum* lines

In this experimental system we crossed two GSH overproducer tobacco lines with an SA-deficient tobacco to test disease resistance and defense responses to viruses, in particular, *Tobacco mosaic virus* (TMV, U1 strain). SA-deficient *nahG* plants (*N. tabacum* cv. Xanthi *nahG*) carrying the transgene salicylate hydroxylase convert SA to catechol (Gaffney et al., 1993). These plants were a gift of Dr. John Ryals, Durham, NC, USA. It is well known that *nahG* plants display enhanced susceptibility to various, mostly biotrophic, pathogens. I used these plants to investigate **whether GSH overproduction can compensate the loss of disease resistance and defense responses due to the lack of SA.** GSH overproducer lines used in this experiment are as follows: *N. tabacum* cv. Burley *CEMK-9* line *(EcSAT* and *EcOASTL* overexpression) *N. tabacum* cv. Burley *TRI-2* line (*EcSAT, EcGSH1* and *SpPCS* overexpression). Serine acetyltransferase (SAT) and O-acetyl-serine (thiol) lyase (OASTL) catalyze the last steps of cysteine biosynthesis, the rate-limiting factor of GSH production in plants. Gamma-glutamylcysteine synthetase (GSH1) is the enzyme that catalyzes the first step of GSH biosynthesis. Phytochelatin synthase (PCS) is involved in the synthesis of phytochelatins. Phytochelatins are oligomers of GSH. These two lines (*CEMK-9*, *TRI-2*) display elevated GSH levels (see in Sirko et al., 2004). The above mentioned GSH overproducer

N. tabacum lines and the GSH-deficient line *AB3-1* were a gift of Prof. Agnieszka Sirko, Institute of Biochemistry and Biophysics, Polish Academy of Sciences. To clarify the role of GSH in SA mediated resistance we crossed SA deficient tobacco (*nahG*) with the two GSH overproducer lines (*CEMK-9, TRI-2*) to obtain F_1 hybrids (*CEMK-9* x *nahG* F_1 and *TRI*-2 x *nahG* F_1). All data on these tobacco lines can be found in **Table 1**. The presence/expression of different transgenes in the tobacco lines and the F_1 generation of crossed plants were assayed by semiquantitative RT-PCR.

Table 1 SA and GSH overproducer/deficient transgenic tobacco lines used in experiments

	Expressed transgene/s	SA, GSH content
Nicotiana tabacum cv. Burley WT	-	Control plant for GSH overproducer lines
N. tabacum cv. Burley *CEMK-9*	*EcSAT, EcOASTL*	GSH overproducer
N. tabacum cv. Burley *TRI-2*	*EcSAT, EcGSH1, SpPCS*	GSH overproducer
N. tabacum cv. Xanthi	-	Control plant for SA deficient line
N. tabacum cv. Xanthi *nahG*	*PpnahG*	SA deficient
N. tabacum cv. Burley *CEMK-9* X *N. tabacum* cv. Xanthi *nahG* F_1	*EcSAT, EcOASTL, PpnahG*	F_1 generation of crossed plants: GSH overproducer and SA deficient
N. tabacum cv. Burley *TRI-2* X *N. tabacum* cv. Xanthi *nahG* F_1	*EcSAT, EcGSH1, SpPCS, PpnahG*	F_1 generation of crossed plants: GSH overproducer and SA deficient
N. tabacum cv. Burley AB3-1	*NtUP9C silencing*	GSH deficient

First, we confirmed altered GSH production in these plants by a High Performance Liquid Chromatography and Mass Spectrometry (HPLC-MS) method as described by (Rellán-Álvarez et al., 2006). With this method we could detect both reduced (GSH) and oxidized (GSSG) glutathione (**Figure 1**).

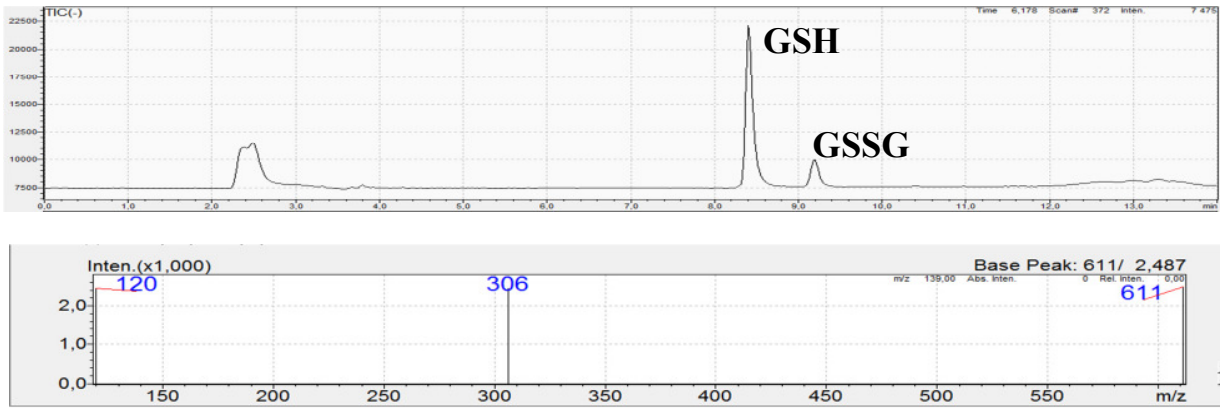


Fig. 1 Chromatogram of reduced (GSH, m/z = 306) and oxidized (GSSG, m/z = 611) glutathione in tobacco obtained by High Performance Liquid Chromatography and Mass Spectrometry (HPLC-MS).

The uninoculated SA deficient *nahG* line showed the lowest glutathione contents among all examined plants. In control cultivars (Burley WT, Xanthi) glutathione contents were higher (60-70 µg/g fresh weight). The glutathione overproducer lines (*CEMK-9*, *TRI-2*) showed even higher levels of GSH as compared to wild type plants, while GSH levels were lower in *AB3-1*. This assay also showed that the amount of the oxidized form of glutathione (GSSG) is ca. 10 % of total glutathione levels in all uninoculated tobacco lines. Mock inoculation did not increase glutathione contents significantly, as compared to uninoculated plants. However, TMV inoculation significantly increased glutathione in GSH overproducer lines (*CEMK9, TRI2*) and the F_1 generation of the crossed plants (*CEMK-9* x *nahG* F_1 and *TRI*-2 x *nahG* F_1 tobaccos). Interestingly, five days after TMV infection levels of oxidized glutathione (GSSG) were unexpectedly higher (i.e. ca. 50 % of total glutathione) in *CEMK-9* x *nahG* F_1 and *TRI-2 x nahG* F_1 tobaccos (**Figure 2**).

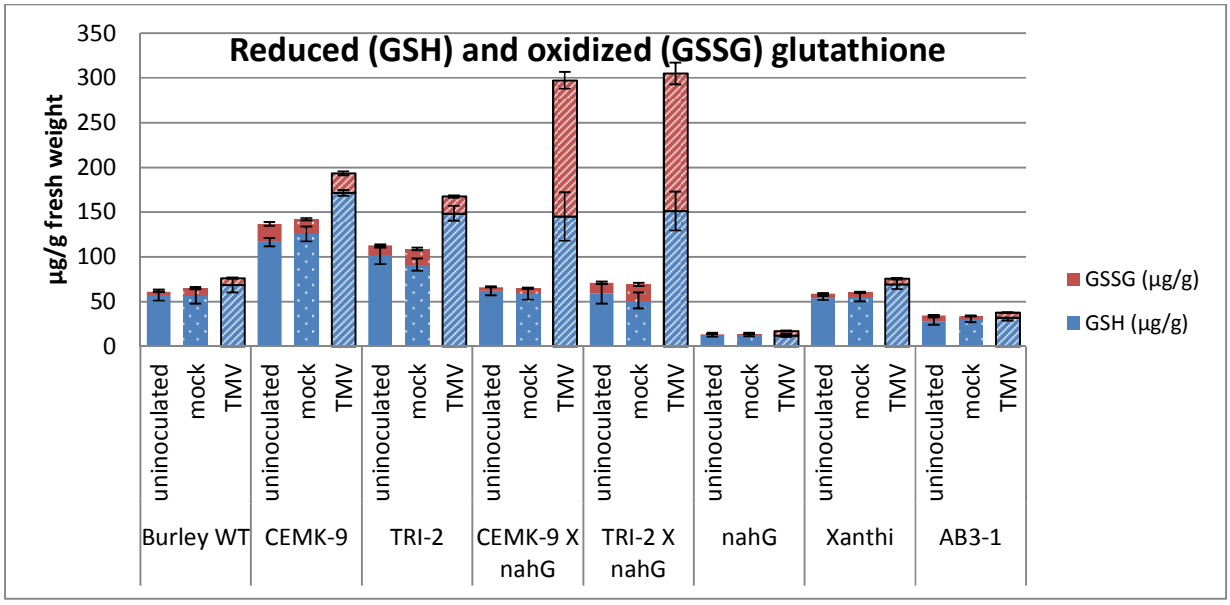


Fig 2. Reduced (GSH) and oxidized (GSSG) glutathione in uninoculated, mock inoculated (mock), and *Tobacco mosaic virus* (TMV) infected tobacco (*Nicotiana tabacum*) lines. Detection of glutathione was performed five days after inoculation. *CEMK-9* and *TRI-2*, GSH overproducer lines; *nahG*, SA-deficient line; *CEMK-9* x *nahG* and *TRI*-2 x *nahG*, F_1 generation plants.

Free and bound (glycosylated) SA contents were determined by High Performance Liquid Chromatography (HPLC) with the kind help of Dr. Gabriella Szalai (AI CAR HAS). Our results showed that levels of both free and bound (glycosylated) forms of SA increase following TMV inoculation, except in *nahG* plants and the F_1 generation of crossed plants containing the *nahG* gene (*CEMK-9* x *nahG* F_1 and *TRI*-2 x *nahG* F_1). It seems that the *nahG* transgene blocked SA accumulation in crossed F_1 plants. Glutathione overproducers (*CEMK-9, TRI-2*) showed higher total SA contents (mostly an increase in bound SA) as compared to the WT control, while SA levels were lower in *AB3-1* (**Figure 3**).

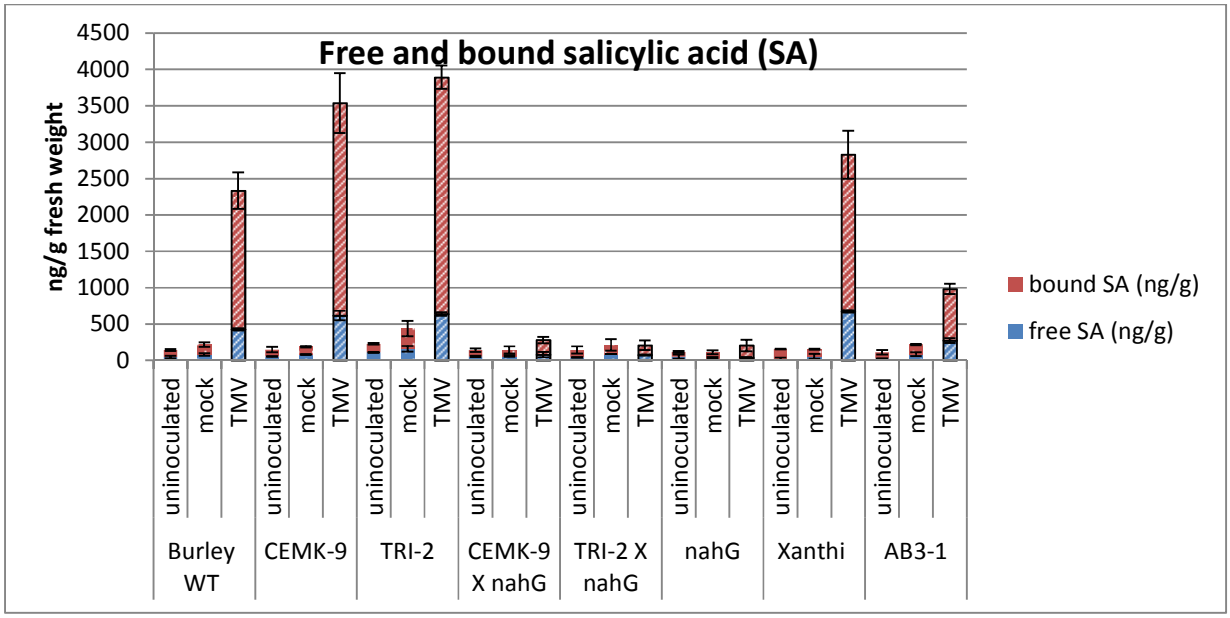


Fig. 3 Free and bound salicylic acid (SA) in uninoculated, mock inoculated (mock), and *Tobacco mosaic virus* (TMV) infected tobacco (*Nicotiana tabacum*) lines. SA detection was performed five days after inoculation. *CEMK-9* and *TRI-2*, GSH overproducer lines; *nahG*, SA-deficient line; *CEMK-9* x *nahG* and *TRI*-2 x *nahG*, F_1 generation plants.

To test the possible role of elevated GSH and SA levels in enhanced resistance to viruses we have inoculated the above mentioned *N. tabacum* cv. Burley lines (wild type, *CEMK-9*, *TRI-2* and *AB3-1*) and F_1 hybrids (*CEMK-9* x *nahG* F_1 and *TRI*-2 x *nahG* F_1) with TMV. As expected, all plants displayed localized necrotic lesions in inoculated leaves within two to three days after virus inoculation. However, there were no pronounced symptom differences among the different plant lines, i.e. lesion diameters and numbers were usually very similar, except for *nahG* plants and hybrid (F_1) plants which contained the *nahG* gene (*CEMK-9* x *nahG* F_1 and *TRI*-2 x *nahG* F_1). In these plants the necrotic symptoms caused by TMV were more severe (lesions coalesced) compared to the other lines which do not contain the *nahG* gene (**Figure 4**).

In order to see if resistance to TMV is manifested on the level of virus accumulation, we monitored expression levels of the TMV gene that encodes the coat protein (TMV CP) by a real time reverse transcription quantitative polymerase chain reaction (real time RT-qPCR) method (**Figure 5**). Five days after TMV inoculation, when local symptoms have fully developed, TMV titers in plant lines with high GSH and SA levels (*CEMK-9* and *TRI-2*) were less than half of that in the wild type. In addition, TMV titers in line *AB3-1* (containing lower than wild type levels of GSH and SA) were more than 1.5 times higher as compared to the wild type. As expected, TMV titers in SA-deficient *nahG* tobacco (cv. Xanthi) were significantly higher as in any other lines investigated. Virus titers in hybrid (F_1) plants which contained the *nahG* gene (*CEMK-9* x *nahG* F_1 and *TRI*-2 x *nahG* F_1) were lower than those in SA-deficient *nahG* plants but higher than in GSH overproducer *CEMK-9* and *TRI-2* lines (**Figure 5**).

Fig. 4 *Tobacco mosaic virus* symptoms five days after inoculation in tobacco (*Nicotiana tabacum*) cv. Burley *CEMK-9* (left) *CEMK-9 x nahG* F_1 (middle) and *N. tabacum* cv. Xanthi *nahG* (right)

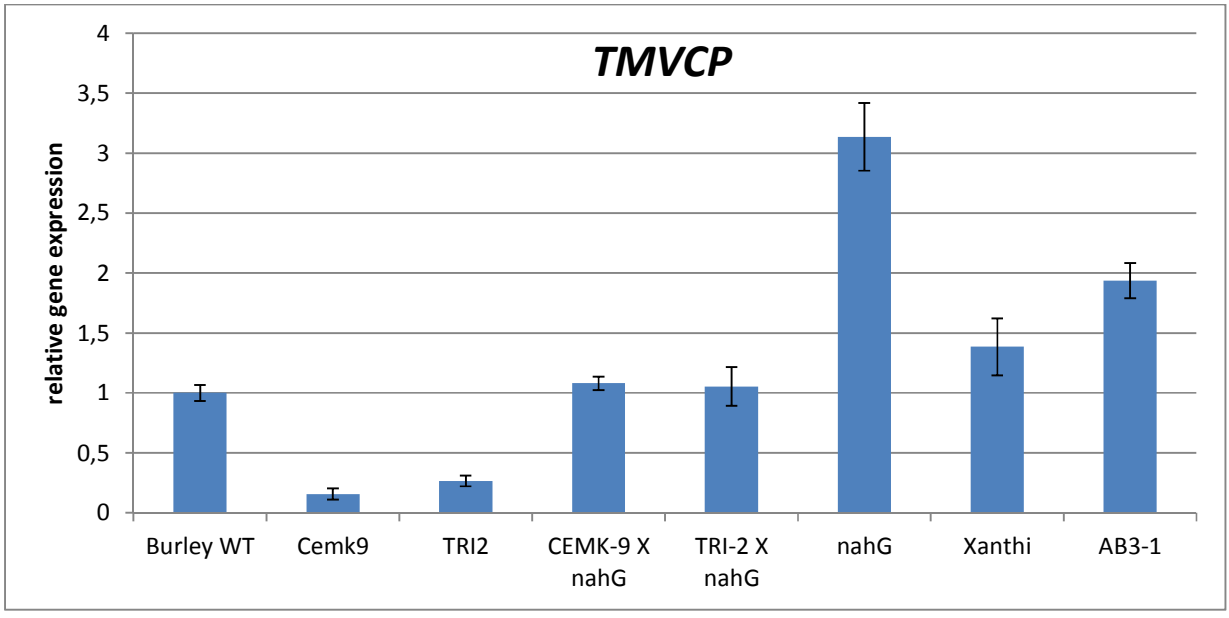


Fig. 5 *Tobacco mosaic virus* coat protein gene (*TMVCP*) expression in different tobacco (*Nicotiana tabacum*) lines five days after inoculation, as detected by a real time reverse transcription quantitative polymerase chain reaction (real time RT-qPCR) method. Expression levels of a tobacco actin gene were used as a reference. *CEMK-9* and *TRI-2*, GSH overproducer lines; *nahG*, SA-deficient line; *CEMK-9* x *nahG* and *TRI*-2 x *nahG,* F_1 generation plants.

As mentioned previously, five days after TMV inoculation, levels of oxidized glutathione (GSSG) were unexpectedly higher (i.e. ca. 50 % of total glutathione) in *CEMK-9 x nahG* F_1 and *TRI-2 x nahG* F_1 tobacco (**Figure 3**). This could be due to the severe oxidative stress and necrosis (plant cell and tissue death)

following virus infection which likely results in mobilization of the relatively high reduced glutathione (GSH) pool. Therefore, high amounts of glutathione are oxidized in order to neutralize reactive oxygen species, the causal agents of oxidative stress and necrosis. It is possible that high amounts of oxidized glutathione (GSSG) induce elevated expression of pathogenesis (stress/defense)-related (PR) genes in these virus-infected F_1 tobaccos in the late stage of TMV infection (5 DPI). Indeed, our results showed elevated expression of the PR gene *NtPRB-1b* as assayed by real time RT-qPCR (**Figure 6**). At 5 DPI, *NtPRB-1b* was highly expressed only in TMV inoculated plants but the expression in crossed F_1 plants was significantly higher as compared to other lines.

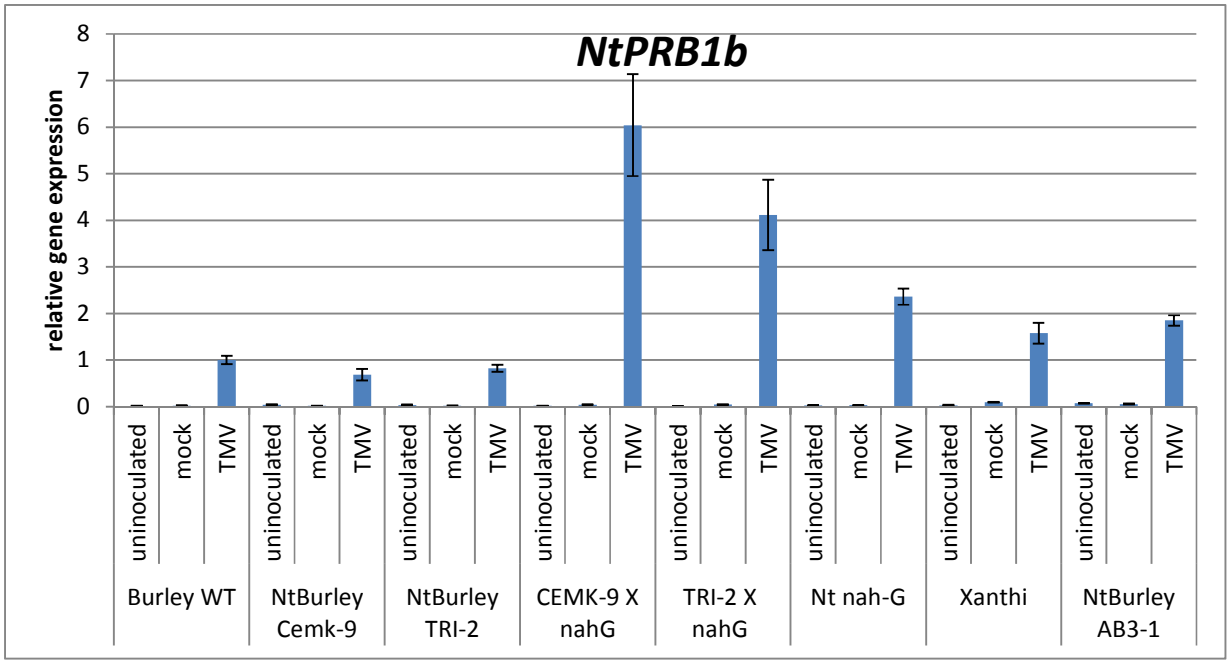


Fig. 6 Expression of the *NtPRB-1b* gene which encodes a basic pathogenesis related protein in tobacco (*Nicotiana tabacum*) plants five days after *Tobacco mosaic virus* (TMV) inoculation. Results are normalized to expression of a tobacco actin gene used as a reference. *CEMK-9* and *TRI-2*, GSH overproducer lines; *nahG*, SA-deficient line; *CEMK-9* x *nahG* and *TRI*-2 x *nahG,* F_1 generation plants.

Moreover we investigated expression of a glutathione S-transferase (GST) gene (*NtGSTPhi*) in TMV infected plants. GSTs have major roles in herbicide detoxification and in endogenous metabolism including functions as glutathione peroxidases to counteract oxidative stress and stress signaling (Dixon et al., 2009, 2011). *NtGSTPhi* expression increased significantly in TMV infected *CEMK-9* x *nahG* F_1, *TRI-2* x *nahG* F_1 and *nahG* tobaccos (**Figure 7**). These changes may correlate with the severe oxidative stress (intensive necrotization) caused by TMV infection in these plants in the late stage of TMV infection (5 DPI).

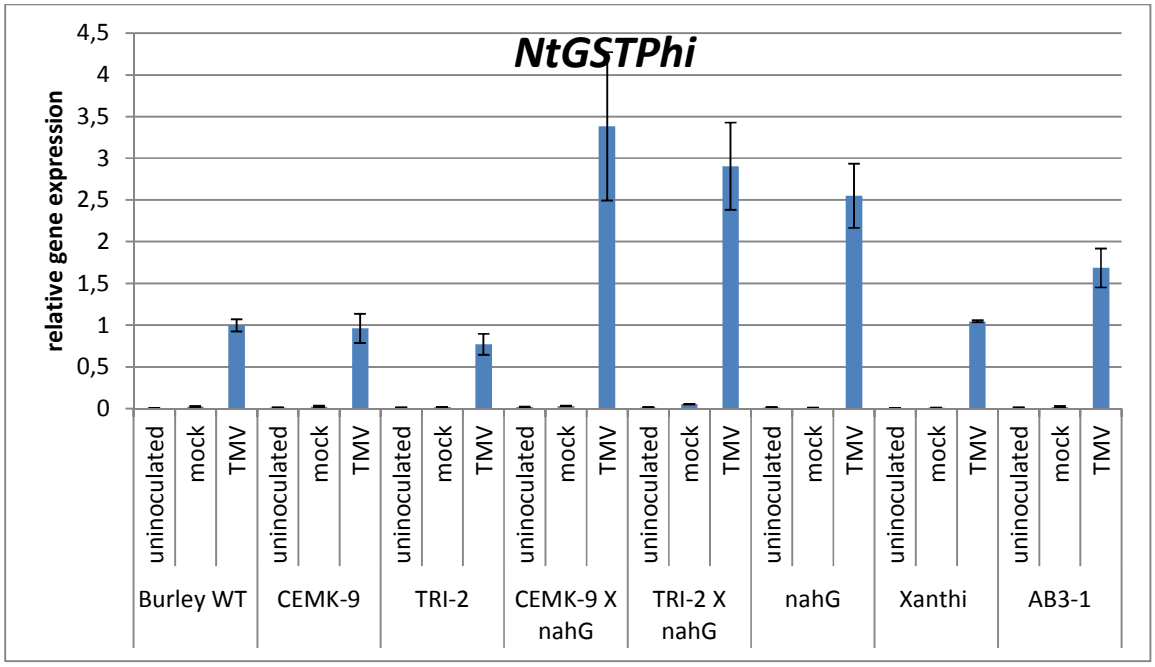


Fig. 7 Expression of a glutathione S-transferase gene (*NtGSTPhi*) in tobacco (*Nicotiana tabacum)* plants five days after *Tobacco mosaic virus* (TMV) inoculation. Results are normalized to expression of a tobacco actin gene used as a reference. *CEMK-9* and *TRI-2*, GSH overproducer lines; *nahG*, SA-deficient line; *CEMK-9* x *nahG* and *TRI*-2 x *nahG,* F_1 generation plants.

Taken together, we have shown that transgenic tobacco plants with higher than normal glutathione contents (*CEMK-9, TRI-2*) display an enhanced resistance to TMV, as compared to wild type controls, while a glutathione deficient tobacco line (*AB3-1*) showed increased susceptibility. SA-deficient *nahG* plants with increased susceptibility to TMV (Gaffney et al., 1993) also showed lower levels of GSH, confirming earlier results (Király et al., 2002). Importantly, when we crossed SA-deficient *nahG* plants with GSH overproducer lines (*CEMK-9, TRI-2*), virus replication was partially inhibited in the F_1 generation, as compared to *nahG* plants. In other words, GSH may at least partially complement the deficiency in SA production and TMV resistance in *nahG* tobacco.

Effects of *in planta* enhancement of GSH biosynthesis on resistance to *Tobacco necrosis virus* in the F_1 generation of a cross between SA-deficient and GSH overproducer *Nicotiana tabacum* lines

In order to generalize our concept on the role of GSH in SA-mediated disease resistance we tested another virus pathogen in the same experimental system as described in the previous chapter. A Hungarian isolate of the *Tobacco necrosis virus* (TNV) E strain was used. We infected the tobacco lines mentioned above with TNV and five days after inoculation evaluated symptoms and virus levels. During infection we also monitored changes of GSH and SA levels. TNV inoculation significantly increased glutathione in all the examined lines, as opposed to TMV inoculation, where a significant increase in

glutathione occurred only in GSH overproducer lines (*CEMK9, TRI2*) and the F_1 generation of crossed plants (*CEMK-9* x *nahG* F_1 and *TRI*-2 x *nahG* F_1). Five days after TNV infection levels of oxidized glutathione (GSSG) were unexpectedly higher (i.e. ca. 50 % of total glutathione) in *CEMK-9* x *nahG* F_1 and *TRI-2 x nahG* F_1 tobaccos (**Figure 8**), the same phenomenon we have experienced after TMV inoculation in the these tobacco lines (see **Figure 2**).

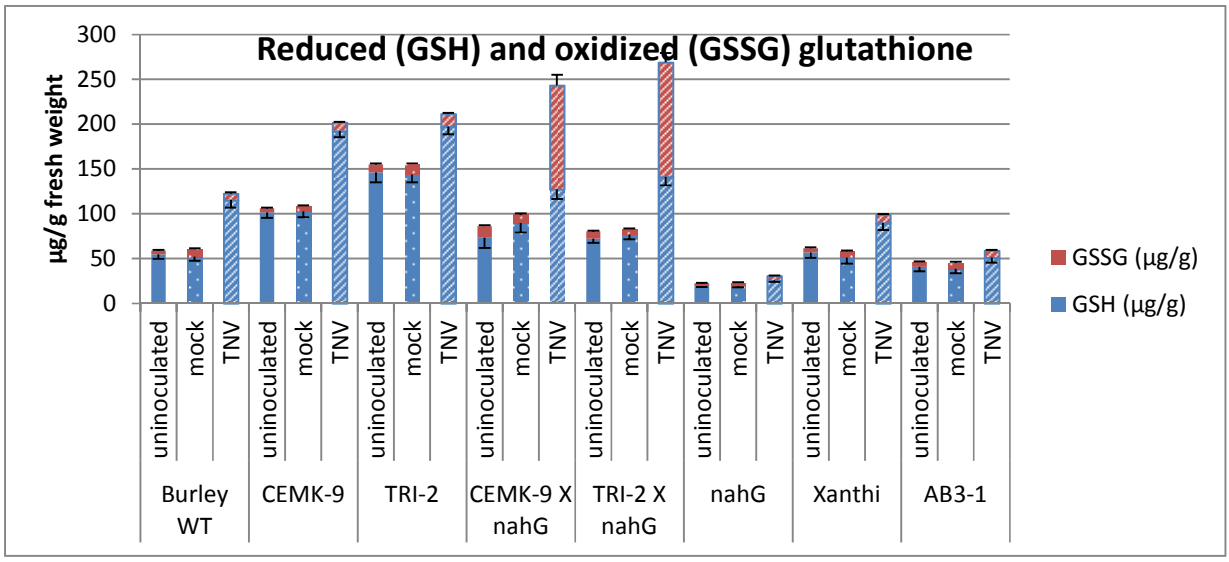


Fig. 8 Reduced (GSH) and oxidized (GSSG) glutathione in uninoculated, mock inoculated (mock), and *Tobacco necrosis virus* (TNV) infected tobacco (*N. tabacum*) lines. Detection of glutathione was performed five days after inoculation. *CEMK-9* and *TRI-2*, GSH overproducer lines; *nahG*, SA-deficient line; *CEMK-9* x *nahG* and *TRI*-2 x *nahG*, F_1 generation plants.

Our results showed that levels of both free and bound (glycosylated) forms of SA increase following TNV infection, except in *nahG* plants and the F_1 generation of crossed plants containing the *nahG* gene (*CEMK-9* x *nahG* F_1 and *TRI*-2 x *nahG* F_1). It seems that the *nahG* transgene blocked SA accumulation in the crossed plants. Glutathione overproducers (*CEMK-9, TRI-2*) displayed higher SA levels; both free and bound SA increased, as compared to WT controls, while SA levels were lower in *AB3-1* (**Figure 9**). Interestingly we obtained the same trend in SA accumulation for both viruses (TMV, TNV) but overall SA levels were lower in TNV infected plants. A possible reason for the lower SA levels following TNV infection could be the fact that tobacco is a nonhost for TNV. Therefore, defense responses other than SA accumulation might be also responsible for TNV resistance in these tobacco lines.

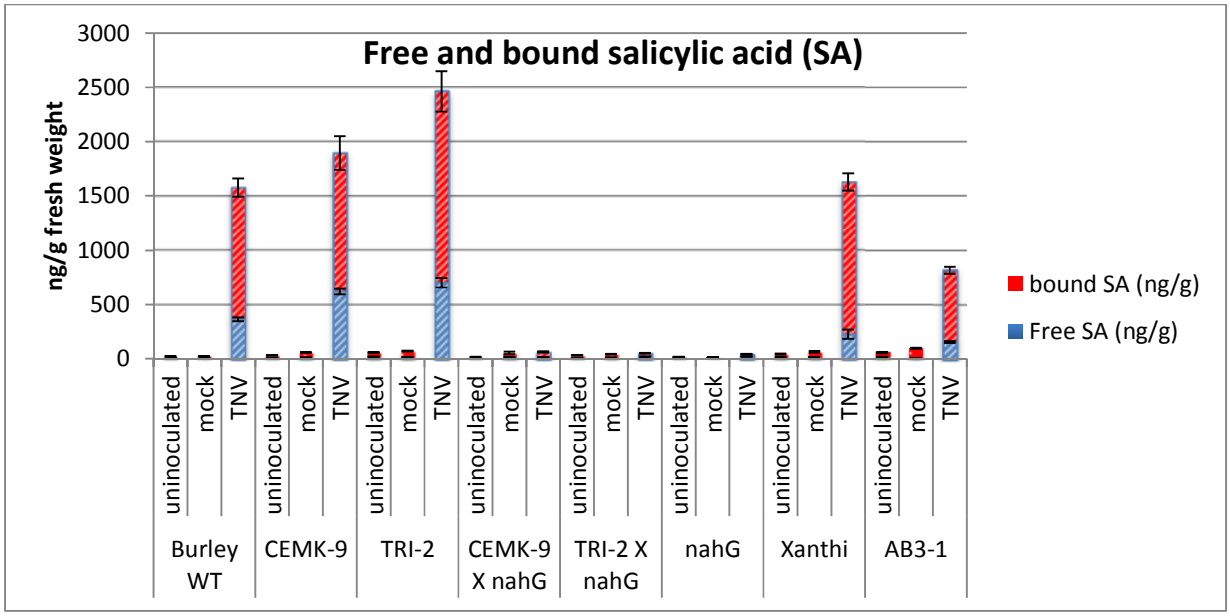


Fig. 9 Free and bound salicylic acid (SA) in uninoculated, mock inoculated (mock), and *Tobacco necrosis virus* (TNV) infected tobacco (*N. tabacum*) lines. SA detection was performed five days after inoculation. *CEMK-9* and *TRI-2*, GSH overproducer lines; *nahG*, SA-deficient line; *CEMK-9* x *nahG* and *TRI*-2 x *nahG*, F_1 generation plants.

Five days after TNV inoculation we evaluated visible symptoms. There were no significant differences in symptoms between the different tobacco lines, except for *nahG* plants and hybrid (F_1) plants which contained the *nahG* gene. As expected, in *nahG* plants the necrotic symptoms caused by TNV were more severe (lesions coalesced) compared to the other lines which do not contain the *nahG* gene. Interestingly, the F_1 generation of crossed plants (*CEMK-9 X nahG* F_1; *TRI-2 X nahG* F_1) displayed distinct symptoms with small lesion size and a high number of lesions, as compared to the *CEMK-9* and *TRI-2* parent (**Figure 10**).

TNV levels were assayed by real time RT-qPCR at 0 hours and two and five days after inoculation. In *nahG* plants, which showed severe necrotic symptoms with coalesced lesions, TNV levels were much higher than in any other lines. TNV accumulation in *CEMK-9 X nahG* F_1 and *TRI-2 X nahG* F_1 was intermediate between that of parents. In GSH overproducer lines (*CEMK-9, TRI-2*), TNV levels were lower than in wild type controls, especially five days after inoculation, and showed a decreasing tendency with time. However, TNV titers in GSH deficient *AB3-1* were similar as in WT controls (**Figure 11**).

Fig. 10 Visible symptoms in tobacco (*N. tabacum*) cv. Burley *CEMK-9* (left) and the F1 generation of *CEMK-9 X nahG* five days after *Tobacco necrosis virus* (TNV) inoculation.

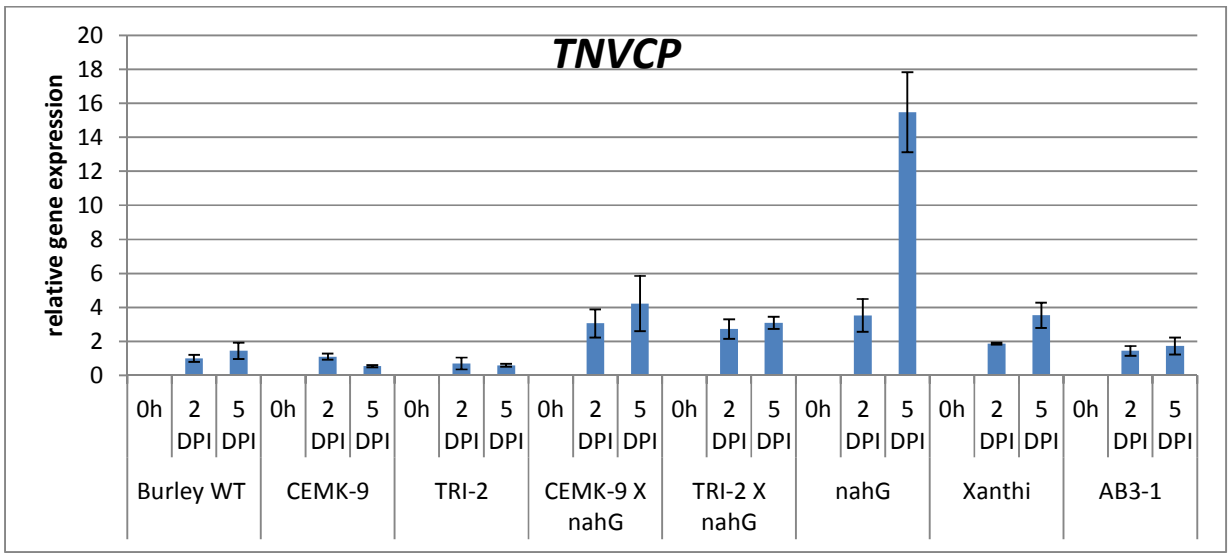


Fig. 11 *Tobacco necrosis virus* coat protein gene (*TNVCP*) expression in different tobacco (*Nicotiana tabacum*) lines zero hours (0h), two and five days (2DPI, 5DPI) after inoculation, as detected by a real time reverse transcription quantitative polymerase chain reaction (real time RT-qPCR) method. Expression levels of a tobacco actin gene were used as a reference. *CEMK-9* and *TRI-2*, GSH overproducer lines; *nahG*, SA-deficient line; *CEMK-9* x *nahG* and *TRI-2* x *nahG,* F_1 generation plants. DPI= days post inoculation

Taken together, we have shown that transgenic tobacco plants with higher than normal glutathione contents (*CEMK-9, TRI-2*) display an enhanced resistance to TNV, as compared to wild type controls, while a glutathione deficient tobacco line (*AB3-1*) did not show any changes in virus levels. We demonstrated that SA-deficient *nahG* plants with increased susceptibility to TMV (Gaffney et al., 1993)

were also more susceptible to TNV. Furthermore, *nahG* plants showed lower levels of GSH, confirming earlier results (Király et al., 2002). Importantly, when we crossed SA-deficient *nahG* plants with GSH overproducer lines (*CEMK-9, TRI-2*), virus replication was partially inhibited in the F_1 generation, as compared to *nahG* plants. In other words, GSH may at least partially complement the deficiency in SA production and TNV resistance in *nahG* tobacco.

Role of artificially added reduced glutathione (GSH) and its precursor, R-2-oxothiazolidine-4-carboxylic acid (OTC), in inducing resistance to *Tobacco mosaic virus* in SA-deficient *nahG* tobacco

In order to strengthen our hypothesis – the role of GSH in SA-mediated disease resistance - we decided to test a different experimental approach. **We wanted to know whether artificially increased GSH contents have the same effects on TMV resistance of SA-deficient tobacco as crossing with GSH overproducer lines?** Various combinations (i.e. different concentrations and timing) of GSH and its precursor, OTC, were applied to SA deficient *nahG* tobacco lines infected with the U1 strain of *Tobacco mosaic virus* (TMV). Infiltration of intact leaves was executed with different concentrations (2 and 4 mM dissolved in pH 6.8 tap water) of GSH and R-2-oxothiazolidine-4-carboxylic acid **(**OTC) (Sigma-Aldrich, Steinheim, Germany) in 8 week old tobacco plants two or three days before TMV inoculation. One half of the intact leaf was infiltrated with GSH or OTC, while the other half with pH 6.8 tap water (control). Two or three days after the GSH/OTC treatments the intact treated leaves were inoculated with TMV.

To access the influence of GSH and OTC treatments on endogenous glutathione levels in uninfected plants, we first infiltrated uninfected, SA-deficient *nahG* tobaccos with 2mM GSH or OTC dissolved in pH 6.8 tap water. We determined the changes of *in planta* glutathione one and two days after infiltration by a spectrophotometric enzymatic recycling assay (Griffith, 1980; Smith, 1985). As a control, we infiltrated *nahG* plants with pH 6.8 tap water. GSH treatments significantly increased total glutathione contents at both time points. Interestingly, however, a large portion of the glutathione pool was oxidized. Importantly, OTC treatments considerably increased the glutathione levels of infiltrated leaves, indicating that OTC is indeed capable of inducing glutathione biosynthesis even in SA-deficient plants (**Figure 12**).

In case of virus inoculated tobaccos we used two different GSH and OTC concentrations (2 and 4 mM) to infiltrate leaves of *nahG* plants two or three days before inoculation with TMV. Three days after virus inoculation glutathione levels were higher in the TMV infected, GSH/OTC treated leaf halves, as compared to water infiltrated controls but significant increases in glutathione contents were detectable only when GSH/OTC was infiltrated two days before virus inoculation (**Figure 13**).

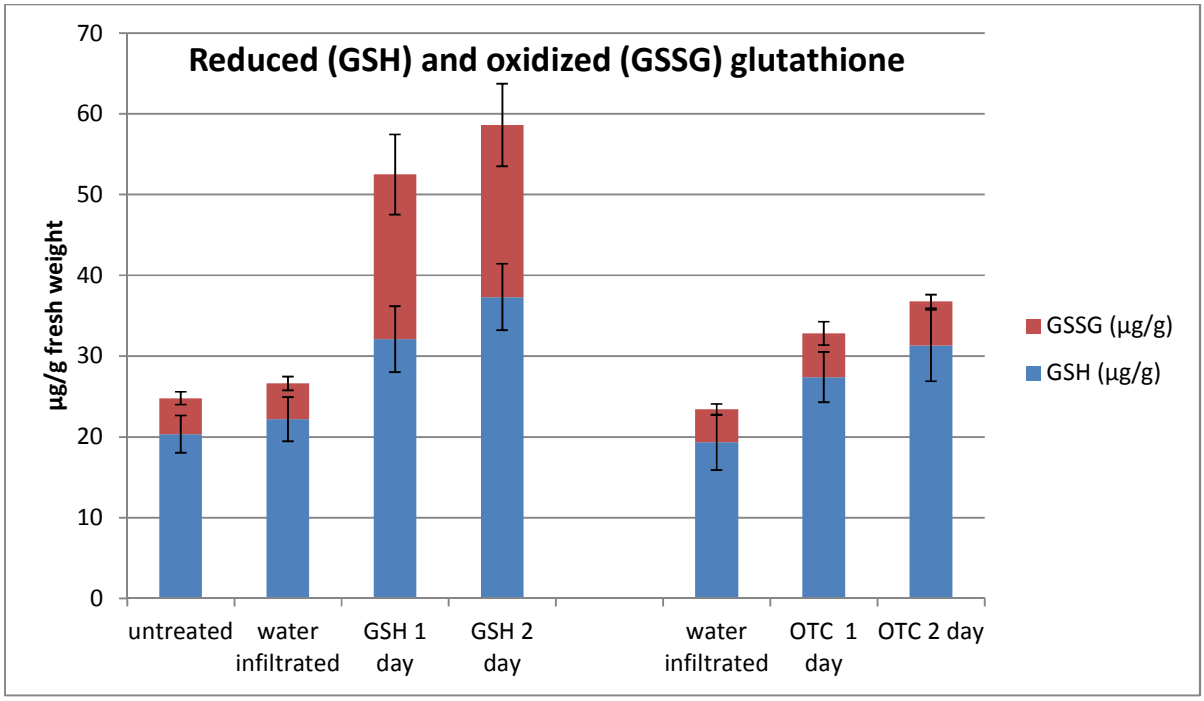


Fig. 12 Detection of *in planta* glutathione in uninfected, SA-deficient *nahG* tobacco one and two days after infiltration with 2 mM GSH or OTC. As a control, we infiltrated *nahG* plants with tap water (water infiltrated). GSH and GSSG: oxidized and reduced glutathione.

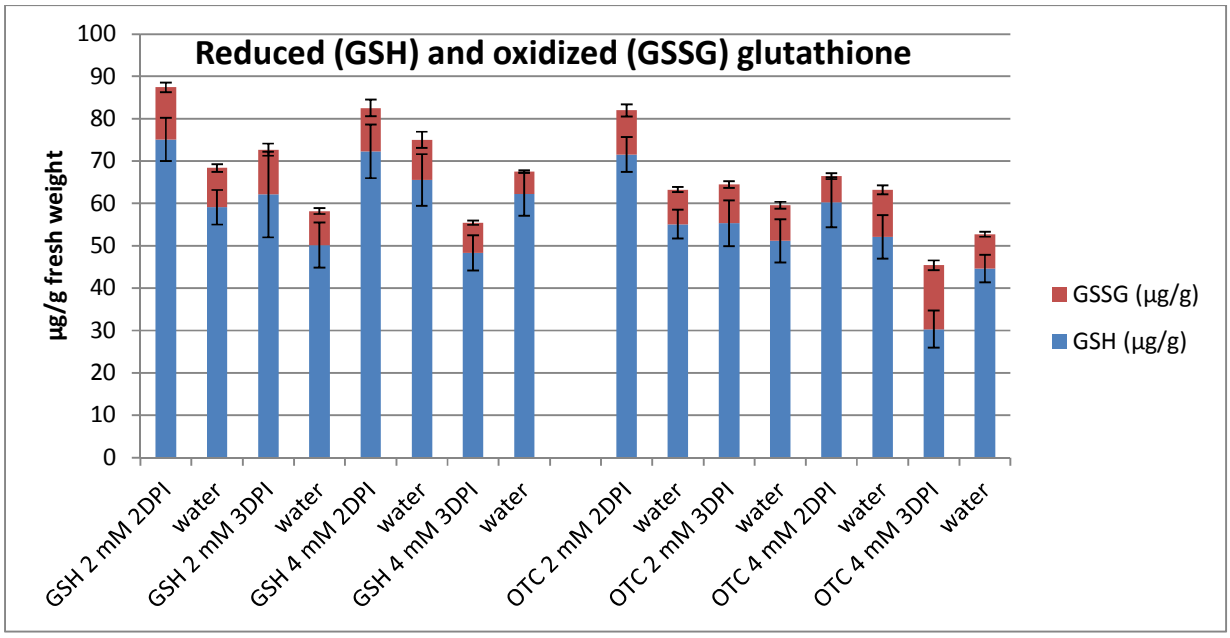


Fig. 13 Detection of *in planta* glutathione in *Tobacco mosaic virus* (TMV) infected SA-deficient *nahG* tobacco three days after inoculation. 2 and 4 mM GSH/OTC were applied 2 or 3 days before TMV inoculation. DPI= days before (pre) inoculation.

Three days after TMV inoculation there were no significant changes in visible symptoms between the GSH/OTC infiltrated and tap water infiltrated leaf halves (**Figure 14**).

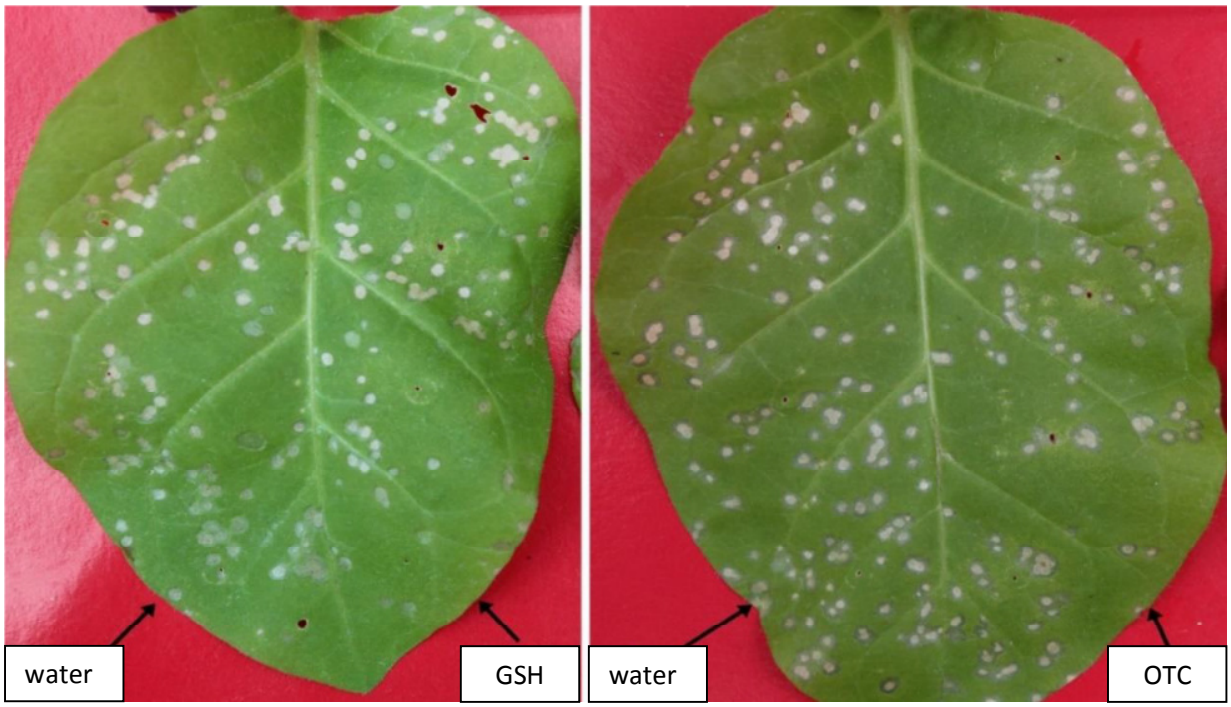


Fig. 14 Symptoms caused by *Tobacco mosaic virus* (TMV) in salicylic acid deficient *nahG* tobacco three days after inoculation. The left side of leaves were infiltrated with tap water (pH 6.8), the right side of leaves were infiltrated with 2 mM GSH/OTC two days before TMV inoculation.

TMV levels were detected by real time RT-qPCR also at three days after inoculation. GSH or OTC treatment (2 mM) applied two days before inoculation successfully decreased the levels of TMV compared to water infiltrated leaf halves. Other GSH and OTC treatments were not able to significantly decrease TMV levels (**Figure 15**). Similar results were obtained in case of TNV infection, demonstrating that the same GSH and OTC treatments can confer resistance to different plant viruses (data not shown).

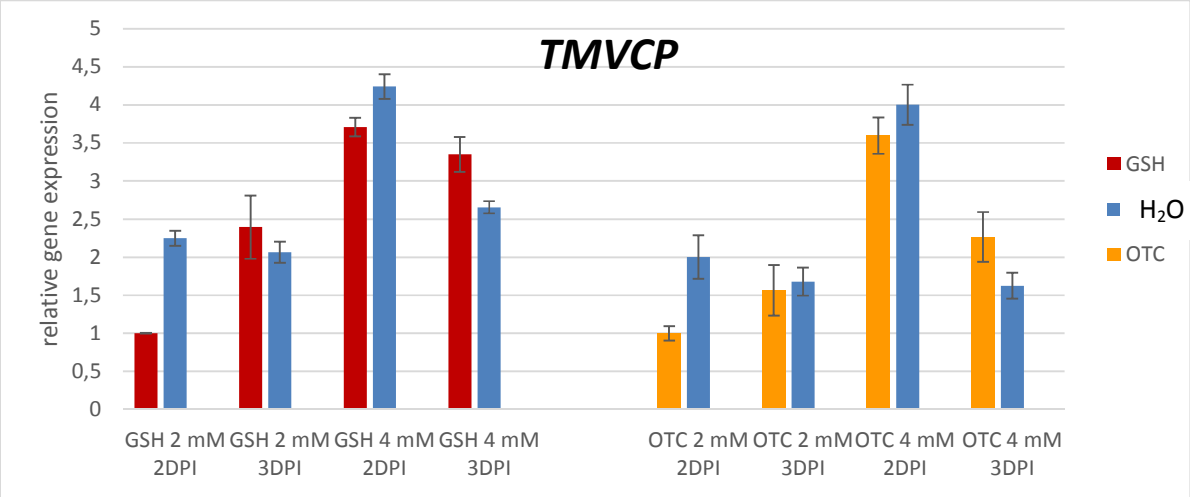
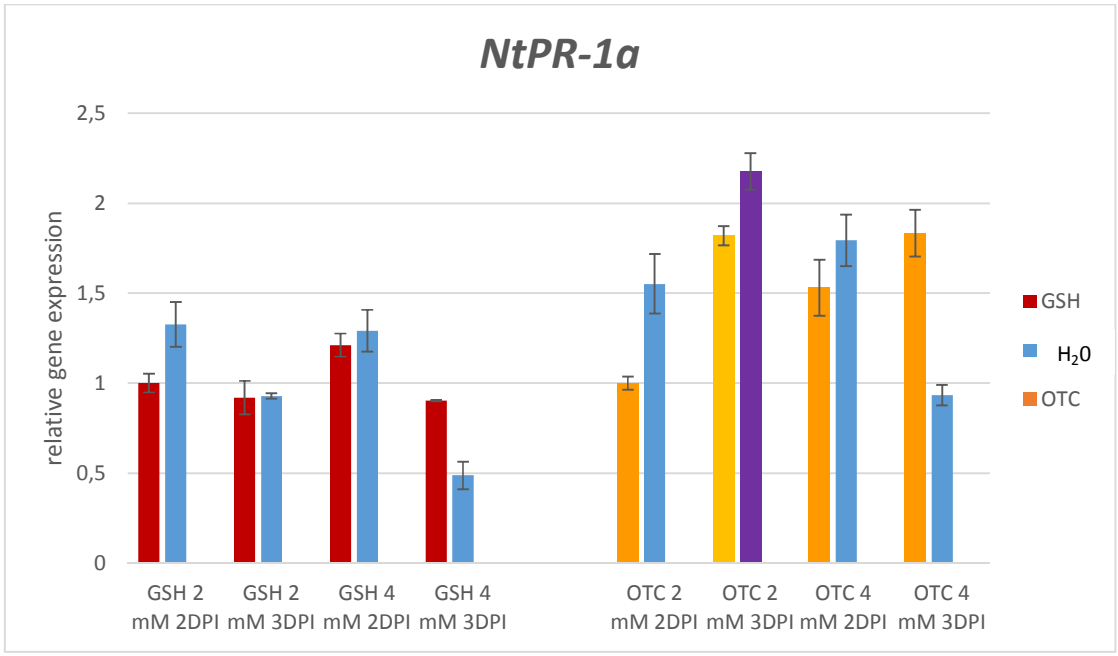
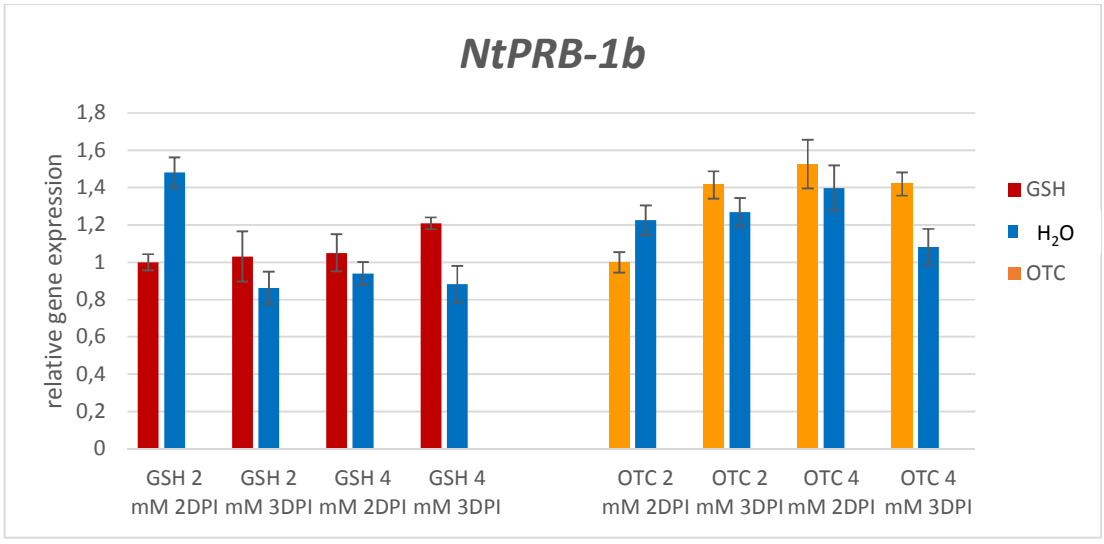


Fig. 15 *Tobacco mosaic virus* coat protein gene (*TMVCP*) expression in SA deficient *Nicotiana tabacum* cv. Xanthi *nahG* leaves three days after inoculation, as detected by a real time reverse transcription quantitative polymerase chain reaction (real time RT-qPCR) method. Leaves were treated with different concentrations (2 and 4 mM) of reduced glutathione (GSH) and R-2-oxothiazolidine-4-carboxylic acid (OTC). Leaves were injected with GSH/OTC two or three days before TMV inoculation. DPI=Days before (pre) inoculation.

Interestingly, TMV levels seem to be inversely correlated with glutathione in infected plants (compare Figure 13 and Figure 15), pointing to a role of artificially elevated levels of glutathione in inducing virus resistance even in SA deficient *nahG* plants. In fact, our results demonstrate that OTC-induced increases in endogenous glutathione can induce TMV resistance not only in wild type but also in SA deficient *nahG* tobacco (Gullner et al., 1999; this study). These results are also in line with previous data showing the inability of SA deficient, TMV-susceptible *nahG* tobacco to maintain a high glutathione pool (Király et al., 2002).

In order to gain a deeper insight into the mechanisms of virus resistance induced by artificially elevated glutathione levels, we assayed expression of different, well characterized stress and defense related genes (*Nt PR1a*, *Nt PRB1b*, *Nt GSTPhi1*, *Nt GSTTau1*) in TMV-infected *nahG* tobacco three days after inoculation. Our results showed that expression of these genes is correlated with virus levels. In samples where GSH/OTC treatments significantly reduce virus levels (2 mM GSH/OTC applied 2 days before inoculation) the examined genes showed a decreased level of expression as compared to control (tap water) inoculated leaf halves. In other words lower virus titers correlate with lower stress/defense gene expression. In samples where the high concentration of GSH/OTC (4mM applied three days before inoculation) may have caused stress responses and virus titers remained high, the defense/stress gene expression increased in the GSH/OTC-treated leaf halves, as compared to water-treated controls.


NtPR-1a
relative gene expression
2,5
2
1,5
1
0,5
0
GSH 2 mM 2DPI
GSH 2 mM 3DPI
GSH 4 mM 2DPI
GSH 4 mM 3DPI
OTC 2 mM 2DPI
OTC 2 mM 3DPI
OTC 4 mM 2DPI
OTC 4 mM 3DPI
GSH
H2O
OTC


NtPRB-1b
relative gene expression
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
GSH 2 mM 2DPI
GSH 2 mM 3DPI
GSH 4 mM 2DPI
GSH 4 mM 3DPI
OTC 2 mM 2DPI
OTC 2 mM 3DPI
OTC 4 mM 2DPI
OTC 4 mM 3DPI
GSH
H2O
OTC

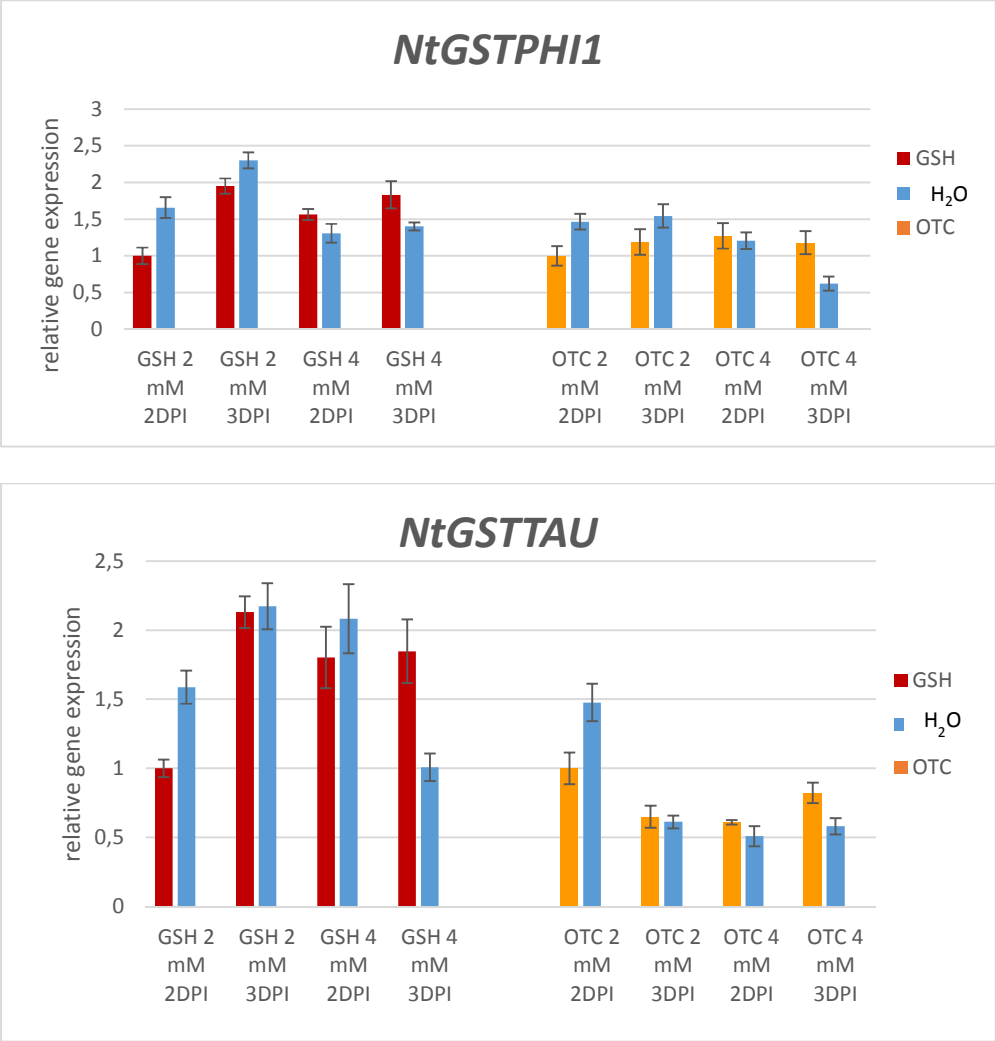


Fig. 16 Expression of stress and defense related genes (*Nt PR1a*, *Nt PRB1b*, *Nt GSTPhi1*, *Nt GSTTau1*) in SA deficient *Nicotiana tabacum* cv. Xanthi *nahG* three days after *Tobacco mosaic virus* (TMV) inoculation. Leaves were treated with different concentrations (2 and 4 mM) of reduced glutathione (GSH) and R-2-oxothiazolidine-4-carboxylic acid (OTC). Leaves were injected with GSH/OTC two or three days before TMV inoculation. DPI=Days before (pre) inoculation Results are normalized to expression of a tobacco actin gene used as a reference.

In summary, our investigations suggest that in virus-infected tobacco high GSH levels can compensate the lack of SA in order to maintain resistance to TMV and TNV. High GSH levels may be achieved either by artificial addition of GSH and OTC solutions (in proper concentrations and timing) or by crossing with GSH-overexpressing plant lines to successfully compensate the lack of SA and maintain resistance to viruses like TMV and TNV.

Role of artificially added reduced glutathione (GSH) and its precursor, R-2-Oxothiazolidine-4-carboxylic acid (OTC), in inducing resistance to a biotrophic fungal pathogen (*Euoidium longipes*) in SA-deficient *nahG* tobacco

Powdery mildews are typical biotrophic pathogens. The powdery mildew fungus *Euodium longipes* can infect *Nicotiana* spp. as first proved in inoculation experiments (Kiss et al., 2008). Later, natural infections in tobacco have been also observed in Europe (Braun et al., 2013). Besides viruses (TMV, TNV), our goal was to test a fungal biotrophic pathogen in our experiments, to clarify if GSH and OTC treatments can maintain resistance also to non-viral pathogens like powdery mildews.

In order to determine the role of GSH and OTC in maintaining/inducing resistance to *E. longipes* in SA-deficient plants, we tried the application of different concentrations of these compounds to SA deficient, *nahG* tobacco lines infected with *E. longipes*. We injected (infiltrated) intact leaves with different concentrations (2 mM, 4 mM) of GSH/OTC solutions dissolved in pH 6.8 tap water. The left side of leaves was injected with pH 6.8 tap water only (control), while the right side of leaves was injected with GSH/OTC. Leaves were infected with *E. longipes* three hours after injection of GSH/OTC. Seven days after inoculation when visible powdery mildew symptoms appeared we evaluated the extent of infection with real-time qPCR. The detection of the fungus was performed by specific primers (PM ITS1, PM ITS2) provided by Dr. Alexandra Pintye (PPI, CAR, HAS). Our results showed that the injection of 2 mM GSH/OTC three hours before inoculation has the best effect in significantly reducing levels of *E. longipes*.

We evaluated the visible syptoms of intact leaves in GSH and OTC-injected *nahG* plants one and two weeks after infection by *E. longipes*. The left side of leaves was injected with pH 6.8 tap water, while the right side of leaves was injected with 2 mM GSH/OTC. One week after inoculation differences in syptom severity could be detected between the GSH/OTC treated and control leaf halves. 2 mM GSH/OTC treatments (right leaf halves) markedly reduced the syptoms of powdery mildew, as compared to water injected controls (left leaf halves). However, two weeks after inoculation no significant differences between GSH/OTC treated and contol leaf halves were detectable (**Figure 17**).

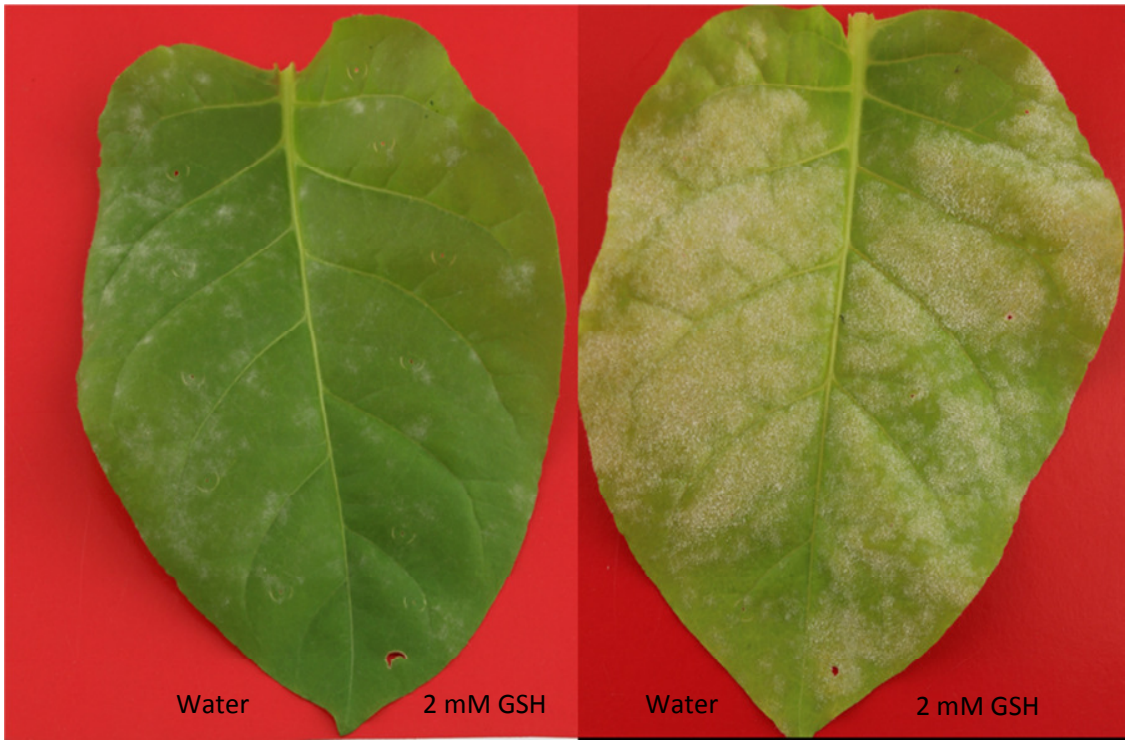


Fig. 17 *Euoidium longipes* symptoms in SA-deficient *nahG* tobacco leaves one (left side) and two (right side) weeks after inoculation. Left leaf halves injected with pH 6.8 tap water, the right leaf halves injected with 2 mM GSH, three hours before inoculation.

In order to clarify if resistance of tobacco to powdery mildew (*E. longipes*) is indeed dependent on salicylic acid (SA), we compared two *N. tabacum* lines: *N. tabacum* cv. Xanthi *nahG*, a SA-deficient tobacco line and *N. tabacum* cv. Xanthi (wild type), where SA production is not damaged. Plants were inoculated with *E. longipes* and after infection, samples were collected to determine powdery mildew biomass at four different time points (2,4,7 and 14 days after inoculation). Our results showed that SA-deficient *nahG* tobaccos are more susceptible to *E. longipes*, as compared to Xanthi wild type (**Figure 18**). The largest difference in fungal biomass appeared one week after inoculation, but after two weeks the difference was still significant. To our knowledge this is the first scientific description of the enhanced susceptibility of SA-deficient tobacco to *E. longipes* .

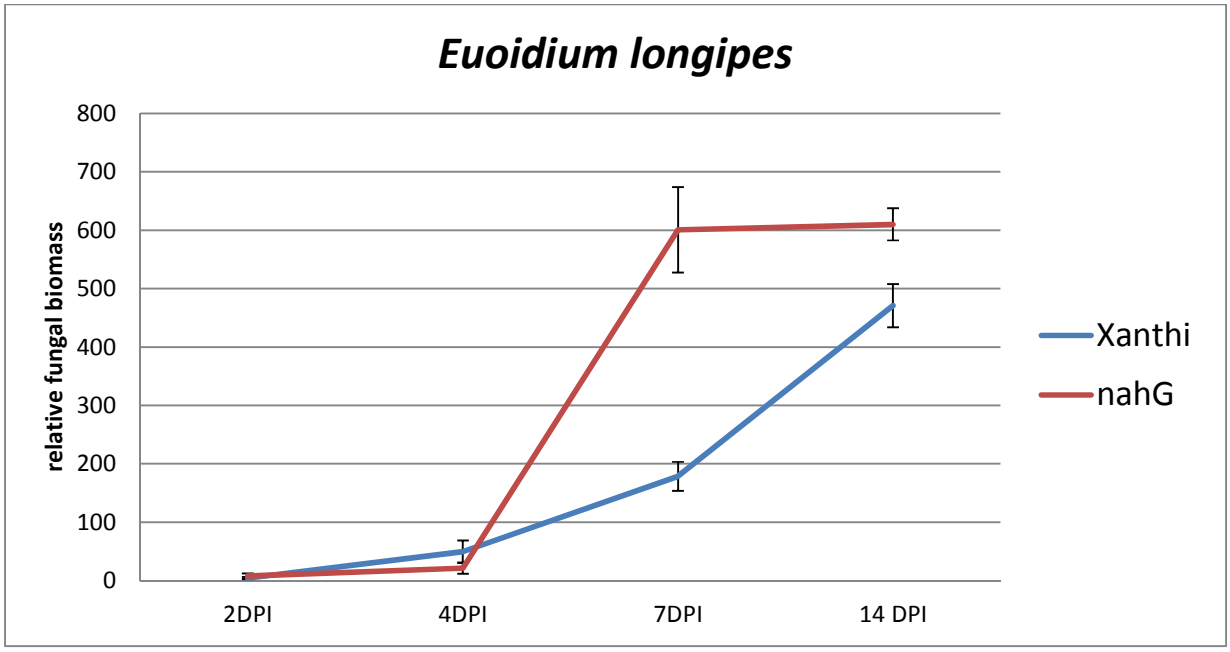


Fig. 18 Relative fungal biomass of *Euoidium longipes* in different tobacco genotypes (*Nicotiana tabacum* cv. Xanthi, *N. tabacum* cv. Xanthi *nahG*), as detected by real-time qPCR. DPI=days post inoculation.

We injected both tobacco lines (SA-deficient *nahG* and wild type cv. Xanthi) with 2 and 4 mM GSH/OTC. Our results showed that injection of 2 mM GSH (three hours before inoculation) dramatically decrease powdery mildew biomass in *nahG* plants one week after inoculation. Two weeks after inoculation the effects of 2 mM GSH on resistance to *E. longipes* are still detectable. In Xanthi (wild type) tobacco which displays normal SA levels and resistance to *E. longipes*, as compared to the *nahG* line, the effect of 2 mM GSH could also induce a significant decrease in fungal biomass one and two weeks after inoculation. On the other hand, injection of 4 mM GSH does not have a significant impact on fungal biomass in any of the tested lines (**Figure 19**). 2 mM OTC treatment decreased the fungal biomass in both lines, however the largest effect on decreasing of fungal biomass in *nahG* plants was observed seven days after infection. 4 mM OTC treatment also decreased the levels of powdery mildew seven and fourteen days after infection in both examined tobacco lines (**Figure 20**).

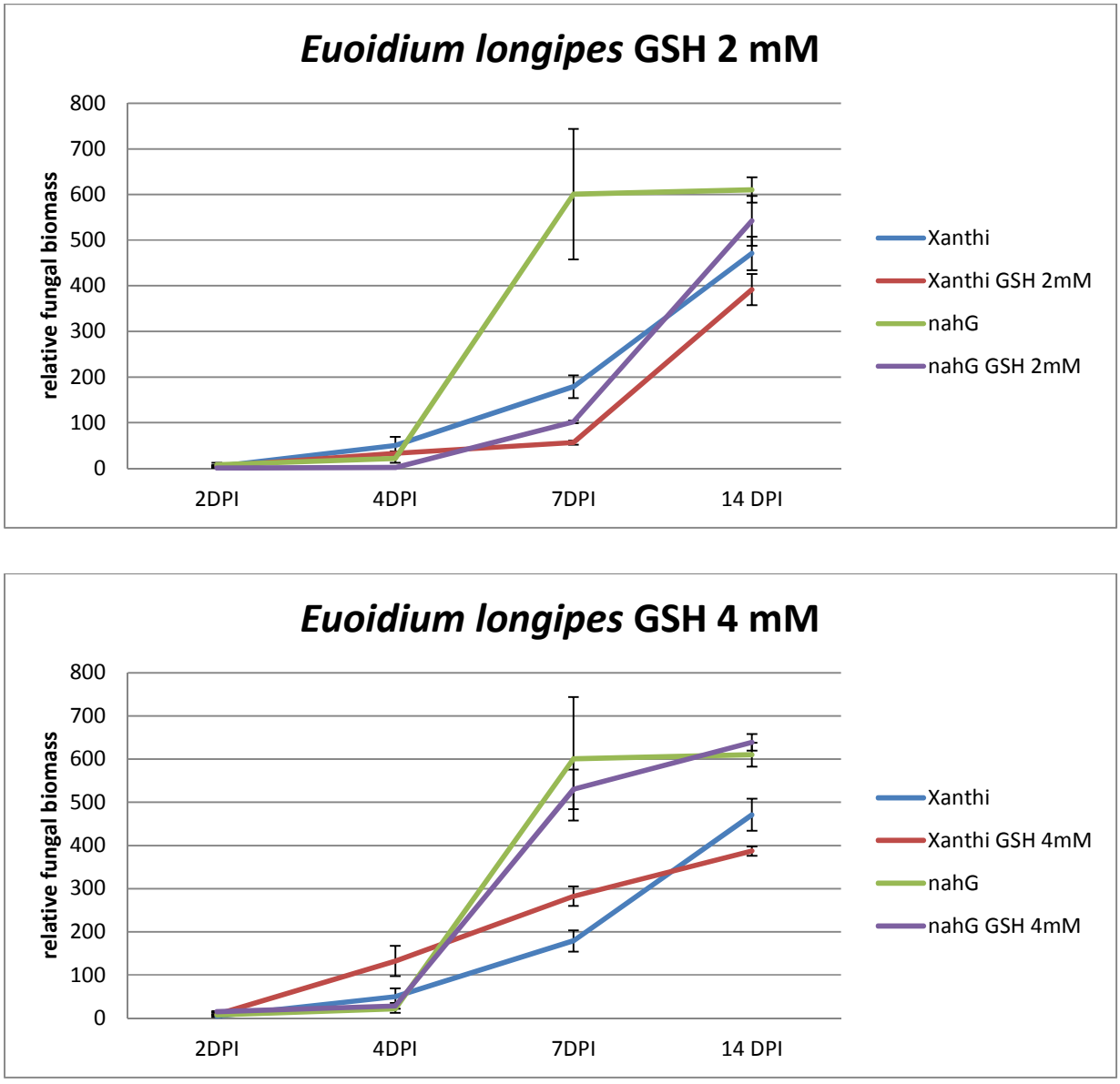


Fig. 19 Effects of 2 and 4 mM reduced glutathione (GSH) treatment (three hours before inoculation) of *Nicotiana tabacum* cv. Xanthi and *N. tabacum* cv. Xanthi *nahG* on *Euoidium longipes* biomass, as detected by real-time qPCR. Powdery mildew detection was performed at four different time points (two, four seven and fourteen days) after inoculation. DPI= days post inoculation.

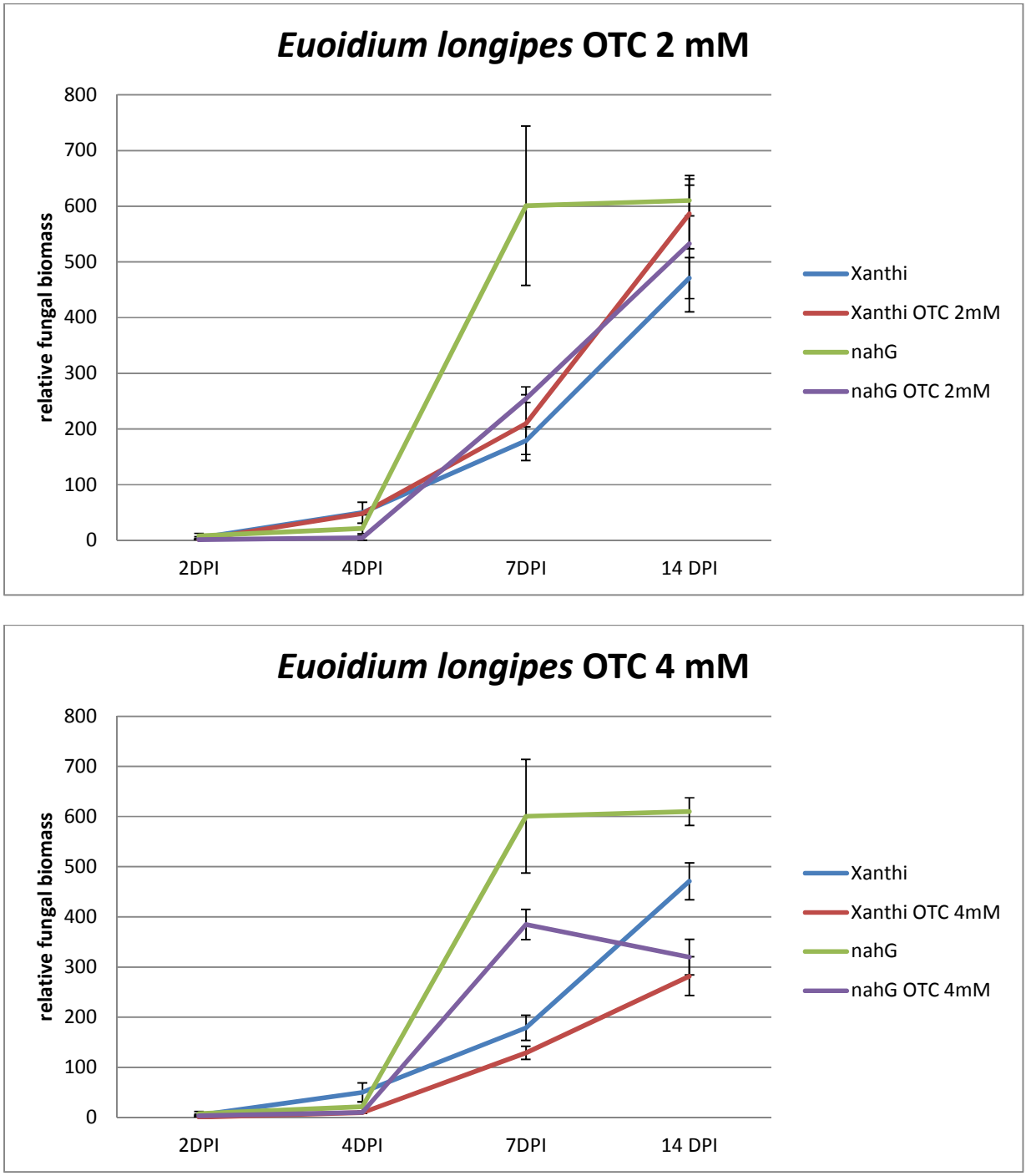


Fig. 20 Effects of 2 and 4 mM R-2-oxothiazolidine-4-carboxylic acid (OTC) treatment (three hours before inoculation) of *Nicotiana tabacum* cv. Xanthi and *N. tabacum* cv. Xanthi *nahG* on *Euoidium longipes* biomass, as detected by real-time qPCR. Powdery mildew detection was performed at four different time points (two, four seven and fourteen days) after infection. DPI= days post infection.

It is known that SA accumulation during pathogen attack triggers the activation of defense genes (e.g. pathogenesis-related /PR/ genes). In *nahG* tobaccos SA cannot accumulate, therefore, expression of PR genes is suppressed in early stages of infection (Ward et al., 1991; Gaffney et al., 1993). We checked *NtPR1a* induction in our experimental system and according to our results, *NtPR1a* is activated in both plant lines but a notable increase during *E. longipes* infection was only detectable in Xanthi (wild type, normal SA accumulation) tobaccos within the first four days following inoculation (**Figure 21**).

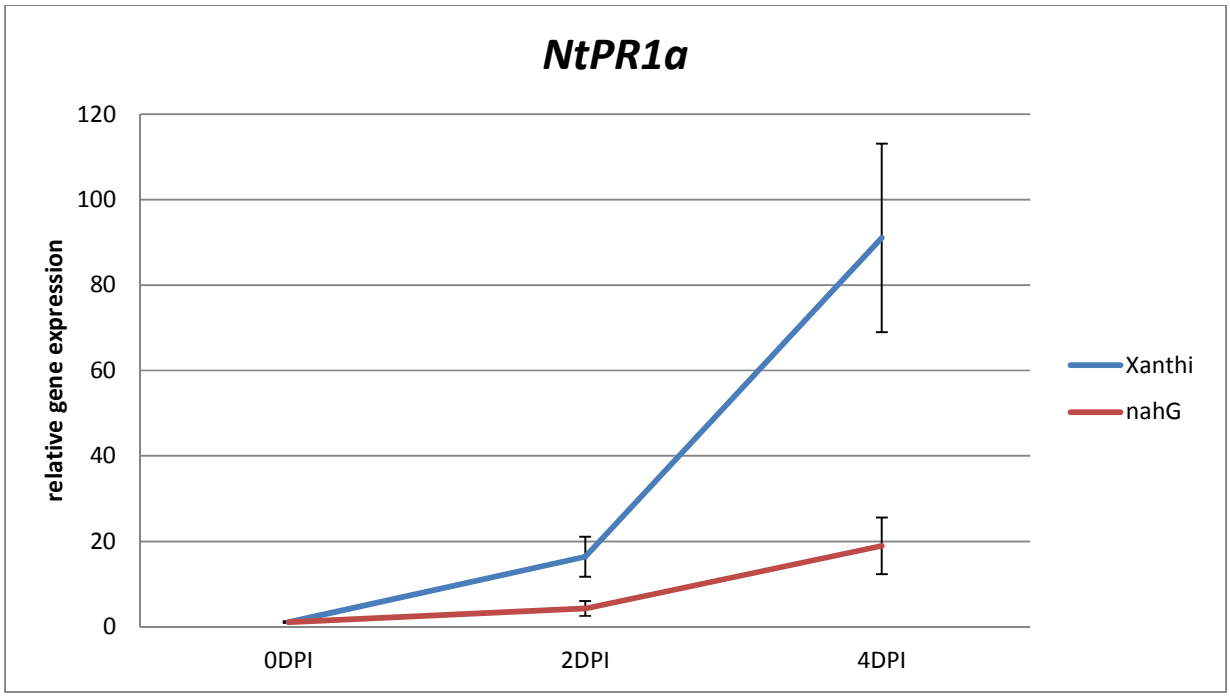


Fig 21 *NtPR1a* expression in *Nicotiana tabacum* cv. Xanthi and *N. tabacum* cv. Xanthi *nahG* plants during the first four days following inoculation with *Euoidium longipes*. DPI= days post infection.

To assess the possible effects of GSH/OTC treatments on PR gene expression in powdery mildew infected *nahG* and Xanthi tobaccos, we assayed *NtPR-1a* gene expression in powdery mildew infected and GSH/OTC treated *nahG* and Xanthi tobaccos immediately after inoculation (0h) and two and four days after inoculation with *E. longipes*. Detection was performed by real time RT-qPCR. Our results showed that GSH/OTC treatments in *nahG* plants couldn't induce *NtPR1a* expression in any given concentrations. However, in Xanthi plants, where SA accumulation is normal, both concentrations (2 mM, 4 mM) of GSH/OTC induced *NtPR1a* expression, especially four days after inoculation (**Figure 22**). These results demonstrate that in early stages of infection SA is necessary for *PR1a* induction and GSH/OTC treatments alone are not sufficient to activate the expression of PR genes. However we proved that GSH/OTC treatments induce resistance to *E. longipes*, suggesting that *NtPR1a* doesn't have a functional role in defense reactions to this particular pathogen.

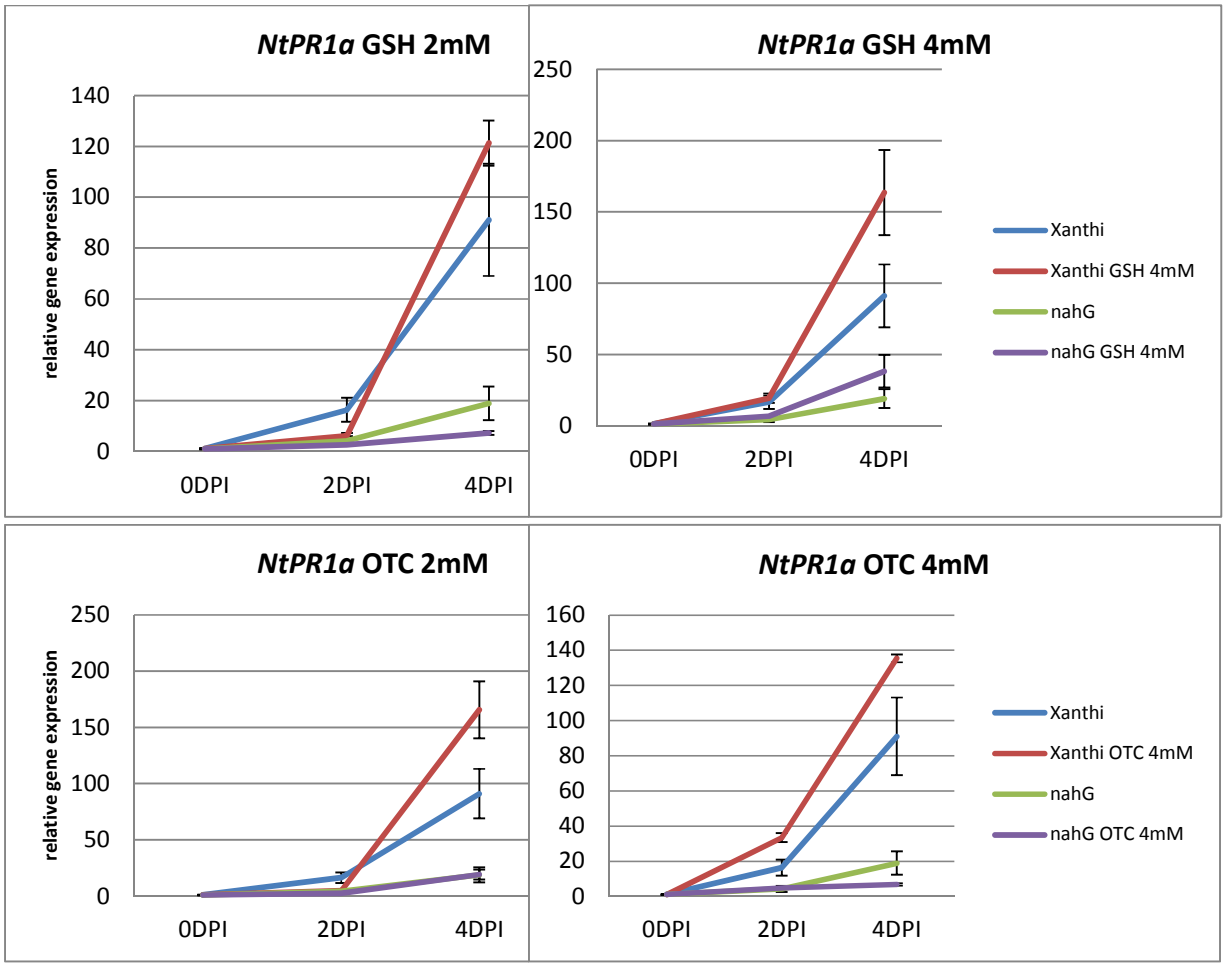


Fig 22 ***Nt*PR1a expression in *Nicotiana tabacum* cv. Xanthi and *N. tabacum* cv. Xanthi *nahG* plants during the first four days following inoculation with *Euoidium longipes*. Plants were treated with different concentrations (2 and 4 mM) of reduced glutathione (GSH) and R-2-oxothiazolidine-4-carboxylic acid (OTC). DPI= days post infection**

Overall we can conclude that GSH/OTC treatments could induce resistance to *Euoidium longipes* in SA-deficient *nahG* tobacco.

Conclusions

Our results showed that increasing GSH contents in an SA-deficient tobacco (*Nicotiana tabacum* cv. Xanthi *nahG*) could restore the injured resistance to different biotrophic pathogens (*Tobacco mosaic virus*; TMV, *Tobacco necrosis virus*; TNV and the powdery mildew *Euoidium longipes*). We investigated the role of GSH in SA mediated resistance by applying two experimental approaches: we either crossed SA deficient tobacco (*nahG*) with GSH overproducer tobacco lines (*N. tabacum* cv. Burley *CEMK-9, TRI-2*)

and used F_1 plants to evaluate possible changes in disease resistance, or artificially infiltrated SA deficient plants with GSH and OTC (a glutathione precursor) prior to virus inoculation/fungal infection.

Previous papers have shown that GSH has a significant impact on SA-mediated plant disease resistance to hemibiotrophic and necrotrophic pathogens (Ghanta et al., 2011 a, b; Kovacs et al., 2015). However, in this project we have demonstrated that high levels of GSH may at least partially restore resistance to biotrophic pathogens (e.g. viruses and powdery mildews) in SA-deficient, susceptible tobacco.

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