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RESEARCH PAPER

Artificial elevation of glutathione contents in salicylic acid-deficient tobacco (*Nicotiana tabacum* cv. Xanthi *NahG*) reduces susceptibility to the powdery mildew pathogen *Euoidium longipes*

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Keywords
Disease resistance; *Euoidium longipes*; glutathione; *NahG*; powdery mildew; salicylic acid; tobacco.

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ABSTRACT

- The effects of elevated glutathione levels on defence responses to powdery mildew (*Euoidium longipes*) were investigated in a salicylic acid-deficient tobacco (*Nicotiana tabacum* cv. Xanthi *NahG*) and wild-type cv. Xanthi plants, where salicylic acid (SA) contents are normal.
- Aqueous solutions of reduced glutathione (GSH) and its synthetic precursor R-2-oxothiazolidine-4-carboxylic acid (OTC) were injected into leaves of tobacco plants 3 h before powdery mildew inoculation.
- SA-deficient NahG tobacco was hyper-susceptible to *E. longipes*, as judged by significantly more severe powdery mildew symptoms and enhanced pathogen accumulation. Strikingly, elevation of GSH levels in SA-deficient NahG tobacco restored susceptibility to *E. longipes* to the extent seen in wild-type plants (*i.e.* enhanced basal resistance). However, expression of the SA-mediated pathogenesis-related gene (*NtPR-1a*) did not increase significantly in GSH or OTC-pretreated and powdery mildew-inoculated NahG tobacco, suggesting that the induction of this *PR* gene may not be directly involved in the defence responses induced by GSH.
- Our results demonstrate that artificial elevation of glutathione content can significantly reduce susceptibility to powdery mildew in SA-deficient tobacco.

INTRODUCTION

Plants produce several defence-related hormones which play important roles in disease resistance. Among these plant hormones, salicylic acid (SA) is a key participant in plant defence responses, since its production is actively induced in resistant plants infected by primarily biotrophic pathogens such as powdery mildews (Malamy *et al.* 1990; Bari & Jones 2009; Klessig *et al.* 2018; Han *et al.* 2019). Furthermore, the exogenous application of SA or its synthetic analogues induces resistance to different pathogens (White 1979; Mandal *et al.* 2009). The indispensable role of SA in plant defence was confirmed by studies carried out in SA-deficient tobacco and *Arabidopsis thaliana*. These transgenic plants express the bacterial *NahG* gene encoding a salicylate hydroxylase enzyme that converts SA to catechol, thus these plants cannot accumulate significant amounts of SA. This defect leads to enhanced susceptibility to viral, fungal and bacterial pathogens (Gaffney *et al.* 1993; Delaney *et al.* 1994; Achuo *et al.* 2004).

Glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) is the most important non-protein thiol compound in plants. It plays a pivotal role in the ascorbate-glutathione cycle as a non-enzymatic antioxidant, and also participates in various detoxification reactions in plant cells due to the highly reactive sulfhydryl group of its cysteine residue (Foyer & Rennenberg 2000; Gullner & Kőmíves 2006; Noctor *et al.* 2012). Furthermore, GSH is also known as a regulator of plant signalling during resistance to pathogen attack. For example, oat powdery mildew (*Blumeria graminis* f. sp. *avenae*) caused a substantial increase in foliar GSH levels in two resistant oat lines but not in a susceptible one 24 h after inoculation (Vanacker *et al.* 1998). In fact, the modulation of GSH levels in plants infected with diverse pathogens and the correlation of GSH with disease resistance has been reported in a number of papers (Fodor *et al.* 1997; Foyer & Rennenberg 2000; Kuźniak & Skłodowska 2004a, b; Zechmann *et al.* 2005; Höller *et al.* 2010; Király *et al.* 2012; Harrach *et al.* 2013; De *et al.* 2018; for reviews see Gullner & Kőmíves 2001, 2006; Gullner *et al.*, 2017b). Cysteine is the rate-limiting amino acid of GSH biosynthesis in plants, and artificial elevation of GSH levels *via* the synthetic cysteine precursor R-2-oxothiazolidine-4-carboxylic acid (OTC) was demonstrated several decades ago (Hausladen & Kunert 1990). OTC pretreatment considerably decreased both the number of necrotic lesions and virus content in *Tobacco mosaic virus* (TMV)-infected tobacco leaf discs, at the same time GSH levels were elevated in the plants studied (Gullner *et al.* 1999). Analogous antiviral effects of OTC treatment were also explored in Styrian oil pumpkin seedlings pretreated with OTC and

inoculated with *Zucchini yellow mosaic virus* (ZYMV). OTC treatment and subsequently elevated GSH content led to a strong decrease in ZYMV content and a suppression of disease symptoms (Zechmann *et al.* 2007). On the other hand, pre-treatment of pea and peach with OTC conferred only partial protection against *Plum pox virus* (PPV) infection, since OTC suppressed symptoms but did not significantly reduce virus level, although *in planta* GSH contents were elevated (Clemente-Moreno *et al.* 2010, 2012, 2013).

It has been demonstrated that both GSH and SA play instrumental roles in plant disease resistance (Mou *et al.* 2003; Ghanta *et al.* 2011). TMV infection in SA-deficient NahG tobaccos resulted in a decreased GSH/GSSG ratio and enhanced virus susceptibility, highlighting the significance of the interplay between SA and GSH during plant disease resistance (Király *et al.* 2002). Intriguingly, the foliar content of H_2O_2, GSH and SA is physiologically coupled, as they can mutually influence each other´s cellular concentrations (Mateo et al., 2006; Han *et al.*, 2013; Gullner *et al.*, 2017b). The main function of the pivotal plant defence regulator Nonexpressor of Pathogenesis-Related protein 1 (NPR1) is the activation of genes encoding pathogenesis-related proteins (PR proteins) in response to SA (Mou *et al.* 2003; Pajerowska-Mukhtar *et al.* 2013; Ding *et al.* 2018). Upon pathogen infection that leads to plant disease resistance, SA accumulation results in activation of the oligomer (inactive) form of NPR1 in the cytoplasm. Activation of NPR1 through redox reactions results in conversion to the monomeric form of the protein which enters the nucleus. Active NPR1 then induces the expression of PR genes through TGA transcription factors. GSH, as a primary determinant of the redox state of plant cells, controls the oligomer–monomer switch of the NPR1 master regulator protein through the reduction of its disulfide bridges (Mou *et al.* 2003; Colville & Smirnoff, 2008; Tada *et al.* 2008; Kovacs *et al.* 2015). Furthermore, in transgenic tobacco plants with high GSH contents, SA levels and resistance to *Pseudomonas syringae* pv. *tabaci* also increased (Ghanta *et al.* 2011). However genetic inhibition of GSH accumulation in *A. thaliana* led to decreased SA levels (Han *et al.* 2013).

The above studies show that GSH plays a major role in SA-mediated plant disease resistance. Our goal was to investigate how exogenously applied GSH and its synthetic precursor OTC influence defence mechanisms in a SA-deficient tobacco that proved to be hyper-susceptible to the powdery mildew fungus *Euoidium longipes*.

MATERIAL AND METHODS

Plant materials, chemical pre-treatments and pathogen inoculation

Nicotiana tabacum L. cv. Xanthi (NN) and the SA-deficient *N. tabacum* L. cv. Xanthi (NN) *NahG* transgenic line (Gaffney *et al.* 1993) were grown under standard greenhouse conditions (18–23 °C; 16 h daylight with 160 $\mu mol \cdot m^{-2} \cdot s^{-1}$ supplemental light for 8 h per day; relative humidity: 75–80%). Artificial elevation of glutathione levels in intact leaves of tobacco plants was performed by infiltration of GSH or OTC solutions 3 h before inoculation on the adaxial leaf side using a hypodermic syringe and needle until the entire leaf lamina was completely water-soaked, as earlier described by Hafez *et al.* (2012). Third and fourth true leaves of 70-day-old tobacco were used to infiltrate aqueous solutions (2 and 4 mM) of reduced glutathione (GSH) or R-2-oxothiazolidine-4-carboxylic acid (OTC) (Cat. No. O6254, Sigma-Aldrich, USA). Right leaf halves were infiltrated with GSH or OTC solutions, while left leaf halves were infiltrated with pH 6.8 tap water as a control. *Euoidium longipes* is a well characterised powdery mildew fungus easily distinguishable from all other powdery mildew anamorphs by having very long conidiophores. *E. longipes* (Hungarian isolate) was maintained separately in infected *N. tabacum* L. cv. Xanthi (NN) *NahG* host plants in a Binder Versatile environmental test chamber (Binder, Tuttlingen, Germany) at 22 °C and 16 h light/8 h dark photoperiod. Conidia from heavily infected *N. tabacum* cv. Xanthi (NN) (*NahG*) tobacco were dusted equally on infiltrated tobacco leaves. The formation of powdery mildew symptoms in leaves was evaluated visually 7 and 14 days after inoculation. Percentage of area covered by powdery mildew symptoms per leaf (Fig. 1) or per leaf half (Fig. 2) was quantified using the ImageJ program (https://imagej.nih.gov/ij/).

Analysis of DNA and RNA

To monitor powdery mildew biomass, total genomic (plant and fungal) DNA was isolated from infiltrated and inoculated leaf halves 2, 4, 7 and 14 days after inoculation. Whole leaf halves were used to quantify powdery mildew biomass. Samples (200 mg fresh leaves) were homogenised in liquid nitrogen followed by extraction with the Plant Genomic DNA Extraction Miniprep System Kit from Viogene (Sunnyvale, CA, USA). Accumulation of the powdery mildew pathogen (*E. longipes*) in inoculated tobacco leaves with or without GSH or OTC treatments was assayed by real-time quantitative PCR (qPCR). For qPCR, a primer pair (PMITS) specific for powdery mildew ribosomal DNA (rDNA) internal transcribed spacer (ITS) region sequences was used: (5′ primer) 5′-TCGGACTGGCCTCAGGGAGA-3′ and (3′ primer) 5′-TCACTCGCCGTTACTGAGGT-3′ as described in Kiss *et al.* (2001). A tobacco actin housekeeping gene (*NtAct*, GenBank X69885) was chosen as internal control, PCR-amplified using the primer pair: (5′ primer) 5′-GCCGTCCTTAGCAGCAGT-3′ and (3′ primer) 5′-ACAAGCAACCCTTCCACC-3′.

The qPCR was performed by using SYBR Green dye (KAPA Biosystems, Wilmington, MA, USA) in a Bio-Rad CFX-96 real-time thermocycler (Bio-Rad, Hercules, CA, USA). PCR reactions were made by mixing 7.5 µl SYBRFAST Readymix Reagent (KAPA Biosystems) with 0.75 µl of each primer (5 µM) and 2.5 µl DNA template ($15\ ng \cdot \mu l^{-1}$). PCR reactions were conducted in triplicate in a total volume of 15 µl with the following program: 1 cycle at 95 °C (5 min), 40 cycles of denaturation at 95 °C (45 s), primer annealing at 60 °C (45 s) and extension at 72 °C (1 min), and 1 cycle at 95 °C (60 s). Melting curve analysis was performed from 65 to 95 °C. Normalisation of fungal DNA levels to plant DNA was carried out with the ΔΔ threshold cycle (Ct) method (Schmittgen & Livak, 2008) using the internal control (reference) mentioned above (tobacco *NtAct* sequences).

Expression of the pathogenesis-related *NtPR-1a* gene (GeneBank D90196) was monitored by reverse transcription real-time polymerase chain reaction (RT-qPCR). Total RNA (plant and fungal) was extracted from leaves of powdery mildew-

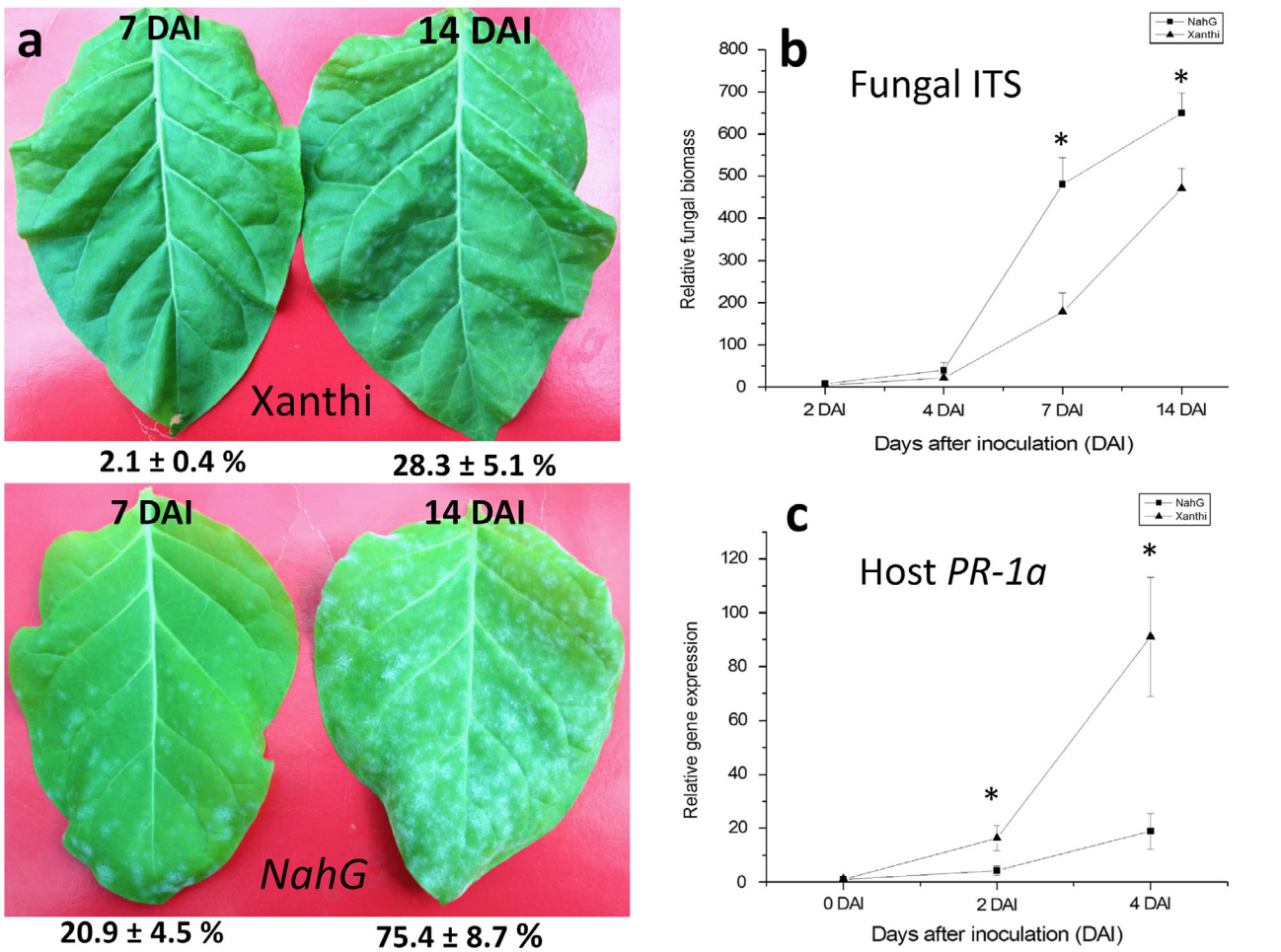

Fig. 1. Powdery mildew symptoms caused by *Euoidium longipes* (a) in wild-type *Nicotiana tabacum* cv. Xanthi (upper panel) and SA-deficient *NahG* (lower panel) tobacco leaves. Symptoms were detected 7 (left leaves) and 14 (right leaves) days after inoculation (DAI). Representative results of three independent experiments are shown. Percentage of area covered by powdery mildew symptoms per leaf was quantified using the ImageJ program. Numbers represent mean ± SD from three independent biological experiments. Detection of *E. longipes* fungal biomass (b) by qPCR with powdery mildew ITS specific primers in wild-type Xanthi and SA-deficient *NahG* tobacco 2 4, 7 and 14 DAI. Assay of pathogenesis-related gene expression (*PR-1a*) (c) by RT-qPCR in wild-type Xanthi and SA-deficient NahG tobacco infected with *E. longipes* at 2 and 4 DAI. Asterisks (*) indicate statistically significant differences between tobacco genotypes. For pair-wise analysis, Student's *t*-test was used ($P = 0.05$).

inoculated tobacco with the Plant Total RNA Extraction Miniprep System Kit (Viogene) following homogenisation of 200 mg fresh leaves per sample in liquid nitrogen immediately after inoculation as well as 2 and 4 days later. Reverse transcription (RT) was done with a RevertAidTM H– cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). To determine *NtPR-1a* expression, qPCR was conducted as described in Höller *et al.* (2010) using the primer pair: (5′ primer) 5′-GCAGATTGTAACCTCGTA-3′ and (3′ primer) 5′-CAATTAGTATGGACTTTCG-3′ for amplifying a *NtPR-1a* (D90196) cDNA fragment. *NtAct* was used as a reference gene, as described above.

Glutathione assays

Total (GSH + GSSG) and oxidised glutathione (GSSG) contents were determined by a spectrophotometric method as described in Knörzer *et al* (1996) with minor modifications. Glutathione levels were assayed from *E. longipes* inoculated NahG and wild-type Xanthi tobacco plants treated with GSH, OTC or water (control) 2, 4, 7 and 14 days after inoculation. Samples from untreated and non-inoculated plants of both tobacco lines were also measured. Leaf tissue samples (0.4 g) were homogenised in liquid nitrogen followed by tissue extraction in 6% w/v stabilised meta-phosphoric acid (Acros Organics, Geel, Belgium). After centrifugation (14,500 g, 20 min, 4 °C), the resulting supernatant was used to determine glutathione contents. One part of the supernatant was used to measure total glutathione. To detect oxidised glutathione (GSSG) we added 2-vinylpyridine (Sigma-Aldrich, USA) to the other part of the supernatant in order to remove GSH.

Statistical analysis

Numerical data represent means of three independent biological experiments ± SD, with three replicates per treatment. Leaf material was pooled from three different plants per biological sample. For pair-wise analysis, statistically significant

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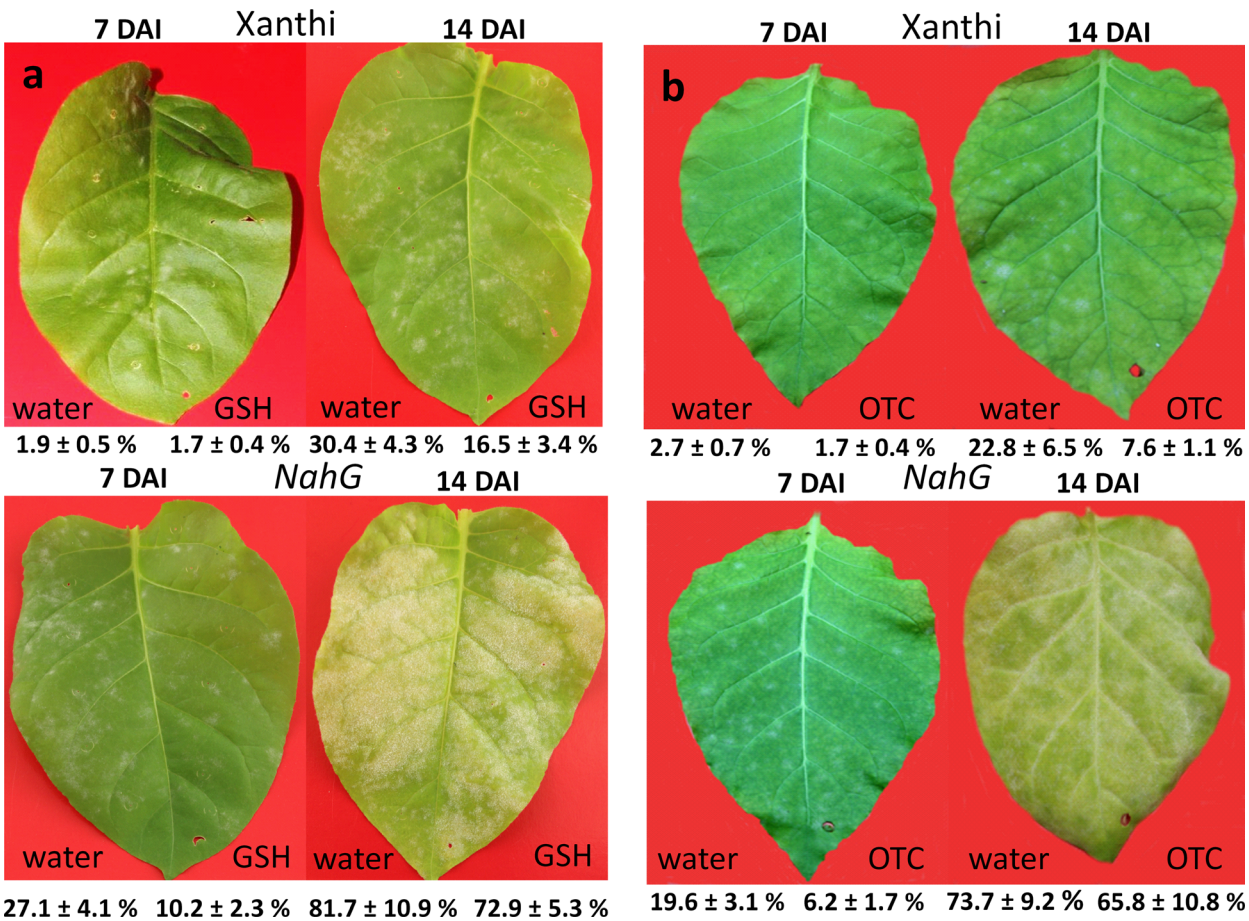

Fig. 2. Effect of artificially added reduced glutathione (GSH) (a) and R-2-oxothiazolidine-4-carboxylic acid (OTC) (b) on symptoms caused by *Euoidium longipes* in wild-type Xanthi (upper panels) and SA-deficient NahG (lower panels) tobacco (*Nicotiana tabacum*) leaves. Symptoms were detected 7 (left pictures) and 14 (right pictures) days after inoculation (DAI). Left leaf halves were infiltrated with pH 6.8 tap water, the right leaf halves with 2 mM GSH or OTC, 3 h before inoculation. Representative results of three independent experiments are shown. Percentage of area covered by powdery mildew symptoms per leaf half was quantified using the ImageJ program. Numbers represent mean ± SD from three independent biological experiments.

differences (in pathogen accumulation, GSH and GSSG contents, *NtPR-1a* gene expression) between tobacco genotypes and between control (water) and GSH/OTC-treated leaf halves of the respective genotype were calculated with Student's *t*-test ($P = 0.05$).

RESULTS

In order to clarify if the resistance of tobacco to the powdery mildew *E. longipes* is indeed dependent on SA, we compared the effects of this fungal infection in two *N. tabacum* lines: *N. tabacum* cv. Xanthi NN (wild type) and *N. tabacum* cv. Xanthi *NahG*, a transgenic SA-deficient tobacco. Leaves were inoculated with *E. longipes*. Evaluation of visible symptoms was executed 7 and 14 days after inoculation. Disease symptoms caused by powdery mildew infection at both time points were more severe in SA-deficient NahG tobacco as compared to Xanthi wild type (Fig. 1a). Quantification of fungal biomass from infected plants was performed by qPCR employing powdery mildew ITS-specific primers. Leaf samples were collected at four different time points: 2, 4, 7 and 14 days after inoculation (DAI). At early time points after inoculation (2 and 4 days) when visible symptoms cannot be detected there were no significant differences in fungal biomass between the two tobacco lines. However, powdery mildew ITS levels were much higher in SA-deficient NahG plants 7 days after powdery mildew inoculation and the difference was still significant after 14 days (Fig. 1b).

Pathogenesis-related (PR) proteins are thought to be involved in plant defence responses (Van Loon & Van Strien, 1999), and it is also known that exogenous SA treatments induces *PR-1a* gene expression in tobacco (Malamy *et al.*, 1990; Delaney *et al.*, 1994). Expression of the *PR-1a* gene is an ideal marker of SA-mediated plant defence responses. Therefore, we investigated *PR-1a* expression by RT-qPCR in SA-deficient NahG and wild-type Xanthi plants in the early stages (immediately after inoculation, and 2 and 4 DAI) of powdery mildew infection. As expected, *PR-1a* gene expression increased significantly in powdery mildew-infected wild-type Xanthi tobacco leaves, however, *PR-1a* transcript accumulation remained at low levels in SA-deficient NahG plants (Fig. 1c).

To understand the role of GSH in plant defence responses in SA-deficient tobacco, we injected aqueous solutions of GSH and OTC at different concentrations into tobacco leaves 3 h

before powdery mildew inoculation. Right leaf halves of intact NahG and Xanthi tobacco plants were injected with 2 and 4 mM GSH or OTC solutions, and as controls we infiltrated the left leaf halves with pH 6.8 tap water (Fig. 2). Visible powdery mildew symptoms caused by *E. longipes* were evaluated 7 and 14 DAI. Overall, powdery mildew symptoms were more severe in NahG plants as compared to Xanthi controls at both time points (Fig. 2). In Xanthi plants only a low amount of mycelium was detectable at 7 DAI both in GSH and water-injected leaf halves (Fig. 2a, upper panel). Similar results were obtained following OTC treatments (Fig. 2b, upper panel). On the other hand, SA-deficient NahG plants displayed much more severe powdery mildew symptoms than wild-type Xanthi tobacco at 7 DAI (Fig. 2). Interestingly, however, GSH or OTC treatments significantly reduced symptom severity at 7 DAI in SA-deficient NahG tobacco (lower panels of Fig. 2a and b, respectively). Fourteen days after inoculation, powdery mildew symptoms became stronger in both tobacco lines. GSH or OTC injection partially suppressed powdery mildew symptoms in Xanthi plants as compared to water-treated leaf halves (upper panels of Fig. 2a and b, respectively); however, in NahG plants symptom severity appeared to be the same in GSH- or OTC-treated and water-infiltrated leaf halves (lower panels of Fig. 2a and b, respectively).

Quantification of powdery mildew biomass in treated and infected plants by qPCR revealed that injection of 2 mM GSH (3 h before inoculation) dramatically decreases powdery mildew biomass in NahG plants 1 week after inoculation (Fig. 3). Two weeks after inoculation the effects of 2 mM GSH on defence against *E. longipes* are still detectable. In Xanthi (wild type) tobacco that displays normal SA levels and susceptibility to *E. longipes* as compared to the NahG line, the effect of 2 mM GSH could also induce a significant decrease in fungal biomass 1 and 2 weeks after inoculation (Fig. 3). On the other hand, injection of 4 mM GSH does not have a significant impact on fungal biomass in any of the tested lines. This could be due to the fact that 7 days after *E. longipes* infection, when visible powdery mildew symptoms appear, glutathione levels are still

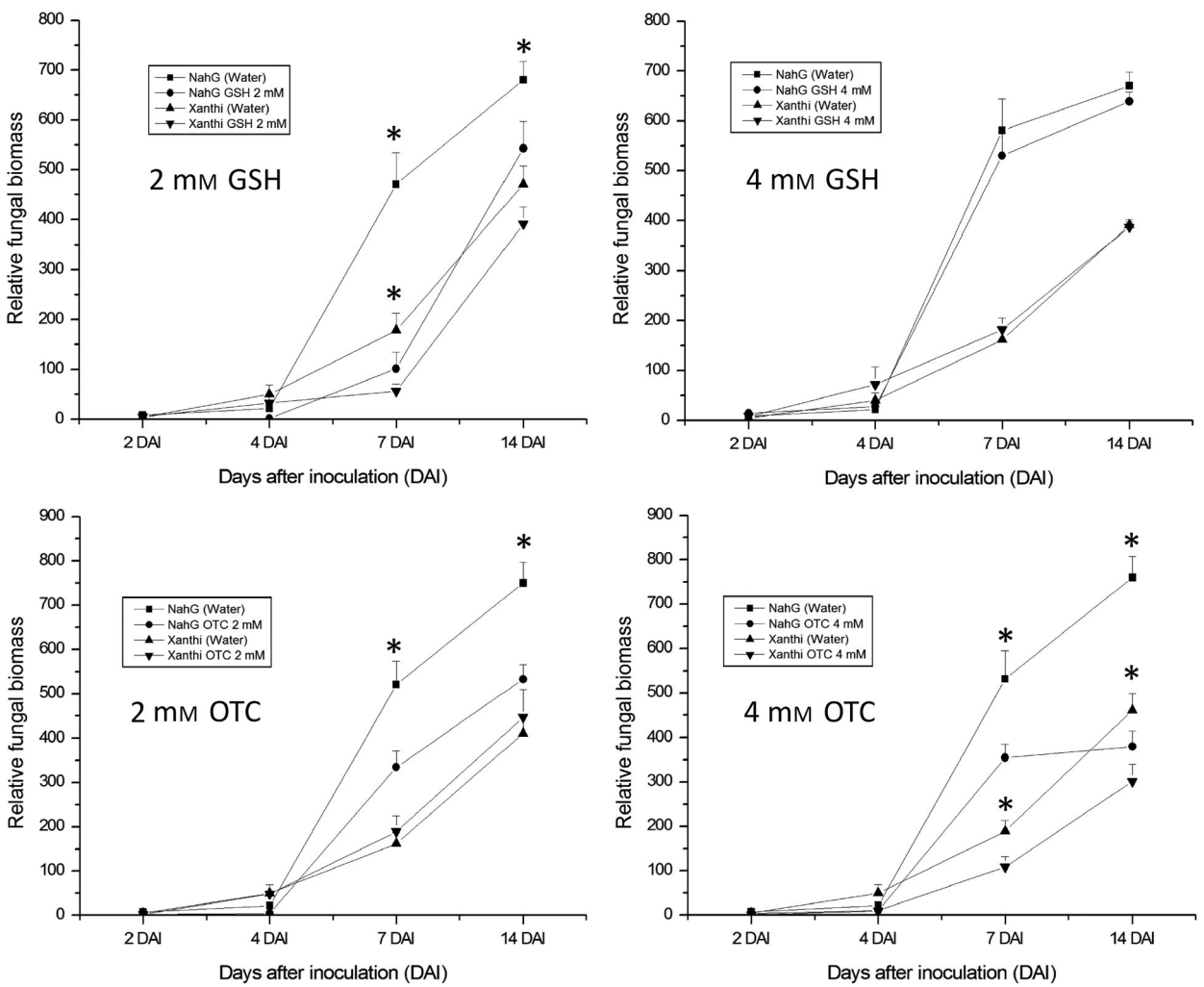

Fig. 3. Effects of artificially added reduced glutathione (GSH) or R-2-oxothiazolidine-4-carboxylic acid (OTC) on fungal biomass of *Euoidium longipes* in wild-type *Nicotiana tabacum* cv. Xanthi and SA-deficient *N. tabacum* cv. Xanthi *NahG* tobacco as detected by qPCR with powdery mildew ITS specific primers. Right leaf halves were infiltrated with 2 and 4 mM GSH or OTC, left leaf halves with pH 6.8 tap water 3 h before fungal inoculation. Samples were collected 2, 4, 7 and 14 days after inoculation (DAI). Asterisks (*) indicate statistically significant differences (Student's *t*-test, $P = 0.05$) between control (water) and GSH/OTC-treated leaf halves of the respective plant genotype.

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high following initial treatment with 2 mM GSH, while in case of 4 mM GSH treatments, glutathione levels have already returned to control levels (Fig. 4). Alternatively, a single infiltration of 4 mM GSH into the apoplast could be deleterious to the plant (Zellnig *et al.* 2000), resulting in a lack of defence against *E. longipes*. Treatments with 2 mM OTC significantly decreased fungal biomass only in the NahG line, the most pronounced effect being observed 7 days after inoculation. However, 4 mM OTC treatments decreased the levels of powdery mildew 7 and 14 days after inoculation in both tobacco lines (Fig. 3), suggesting that this OTC concentration is not phytotoxic. In fact, Gullner *et al.* (1999) have demonstrated that even a continuous supply of 5 mM OTC to tobacco leaf disks is not toxic and able to induce resistance to *Tobacco mosaic virus* (TMV).

In parallel experiments, contents of total glutathione (GSH + GSSG) and oxidised glutathione (GSSG) were monitored in tobacco leaves treated with GSH, OTC and water as control during *E. longipes* infection. Treatments with 2 mM GSH significantly increased GSH and GSSG contents in both tobacco lines (SA-deficient NahG and wild-type Xanthi tobacco) at 2, 4 and 7 DAI (Fig. 4). Interestingly, 4 mM GSH increased plant glutathione contents only at 2 and 4 DAI, but generally higher glutathione contents were detectable as compared to 2 mM GSH treatments (Fig. 4). OTC treatment induced foliar glutathione levels in a similar manner, but the quantity of glutathione was lower as compared to the corresponding GSH treatments. Treatment with 2 mM OTC markedly elevated foliar glutathione levels 2, 4 and 7 DAI, whereas treatment with 4 mM OTC successfully increased glutathione levels at all four investigated time points (Fig. 5). Control treatments with pH 6.8 tap water had no significant effects on glutathione contents as compared to untreated, non-inoculated controls (Figs 4 and 5).

Interestingly, we observed that GSH treatments strongly increased the ratio of oxidised glutathione (GSSG) to total glutathione in both SA-deficient and wild-type plants infected with *E. longipes* (Fig. 4, Figure S1). In OTC-treated leaves of Xanthi and NahG plants, GSSG accumulation was not significant (Fig. 5, Figure S1). GSH treatments increased the GSSG/total glutathione ratio to a similar extent also in uninfected plants (data not shown), indicating that the increase in GSSG levels is not related to powdery mildew infection. To further evaluate this phenomenon, we directly compared the ratio of oxidised glutathione to total glutathione in treatment combinations including only GSH- or OTC-treated leaf halves (Figure S1). The ratio of GSSG in GSH-treated plants varied between 4–32% of total glutathione and was particularly high at 2 and 4 DAI. In contrast, OTC treatment did not significantly induce elevation of the GSSG ratio (4–12%).

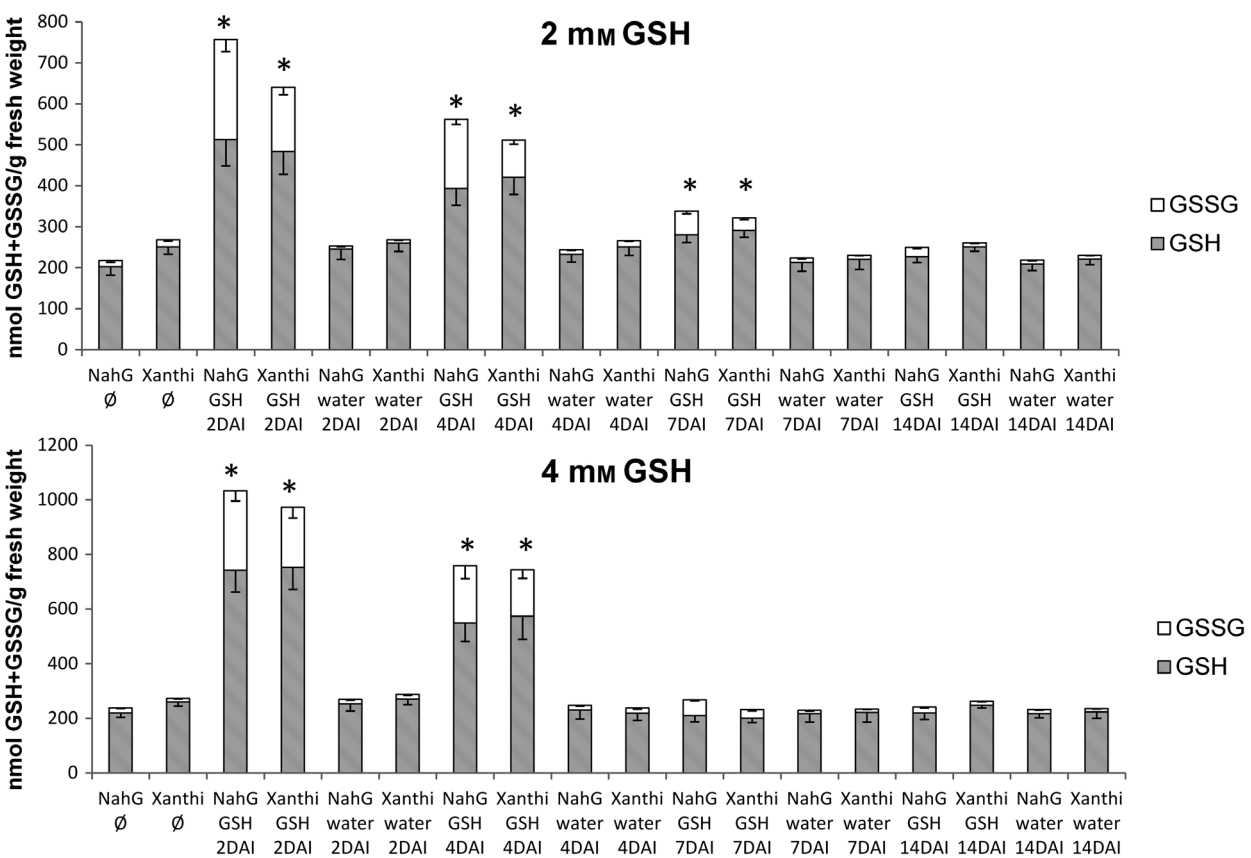

Fig. 4. Reduced (GSH) and oxidised (GSSG) glutathione contents in SA-deficient *Nicotiana tabacum* cv. Xanthi *NahG* and wild-type control *N. tabacum* cv. Xanthi tobacco during *Euoidium longipes* infection. Right leaf halves were infiltrated with 2 and 4 mM GSH, left leaf halves with pH 6.8 tap water 3 h before inoculation. Samples were collected 2, 4, 7 and 14 days after inoculation (DAI). Ø = untreated, non-infected tobacco. Asterisks (*) indicate statistically significant differences (Student's *t*-test, $P = 0.05$) between both GSH and GSSG levels of control (water) and GSH-treated leaf halves of the respective plant genotype.

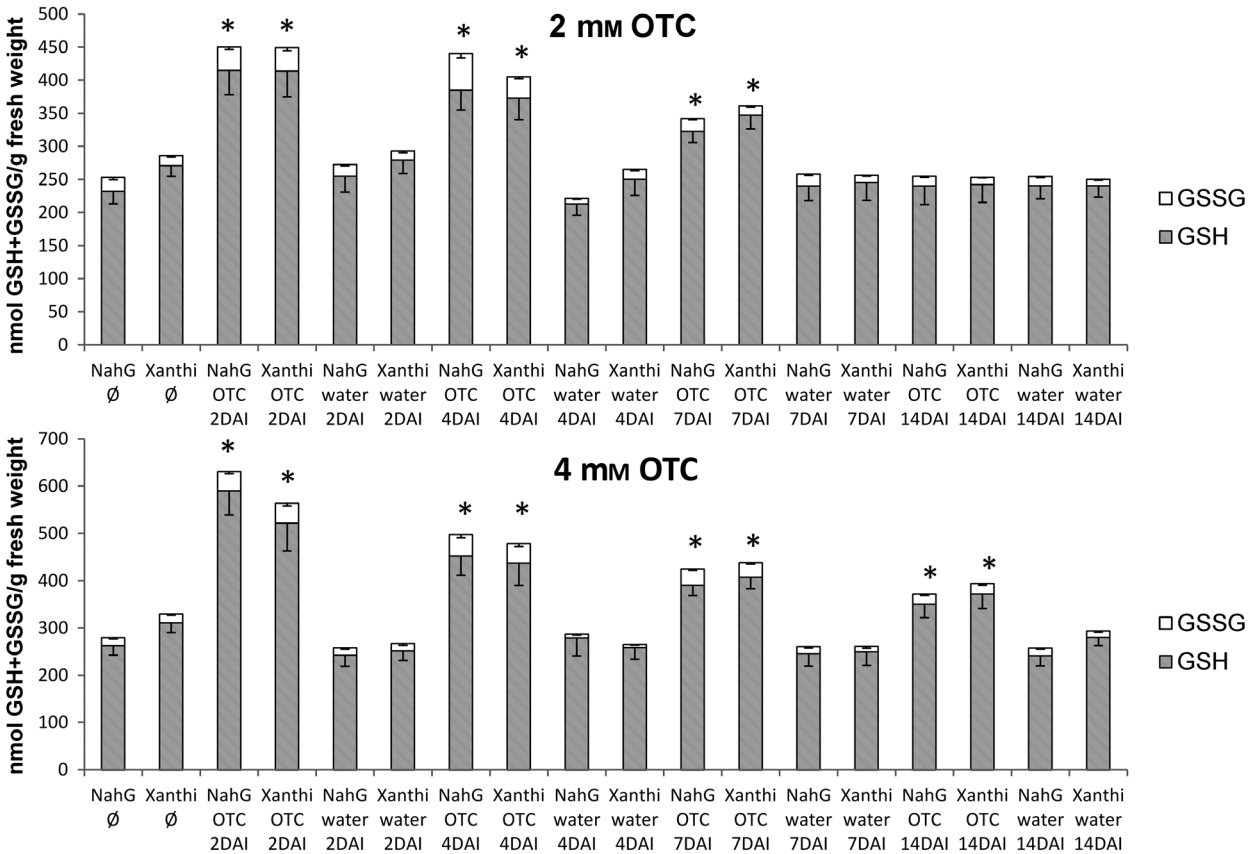

Fig. 5. Reduced (GSH) and oxidised (GSSG) glutathione contents in SA-deficient *Nicotiana tabacum* cv. Xanthi *NahG* and wild-type control *N. tabacum* cv. Xanthi tobacco during *Euoidium longipes* infection. Right leaf halves were infiltrated with 2 and 4 mM R-2-oxothiazolidine-4-carboxylic acid (OTC), left leaf halves with pH 6.8 tap water 3 h before inoculation. Samples were collected 2, 4, 7 and 14 days after inoculation (DAI). Ø = untreated, non-infected tobacco. Asterisks (*) indicate statistically significant differences (Student's *t*-test, $P = 0.05$) between both GSH and GSSG levels of control (water) and OTC-treated leaf halves of the respective plant genotype.

Remarkably, the ratio of GSSG was significantly higher at almost every time point in GSH-treated SA-deficient NahG plants, as compared to wild-type Xanthi (Figure S1).

As mentioned above, in SA-deficient NahG tobacco leaves GSH and OTC treatments suppressed hyper-susceptibility to *E. longipes* to the extent seen in wild-type plants (Figs 2 and 3). To assess the possible effects of GSH or OTC treatment on defence-associated, pathogenesis-related (*PR*) gene expression in powdery mildew-infected NahG and wild-type Xanthi tobaccos, we assayed *PR-1a* gene expression immediately after inoculation (0 h) and 2 and 4 days after inoculation with *E. longipes*. In Xanthi plants, where SA accumulation is normal, both concentrations (2 and 4 mM) of GSH or OTC markedly induced *PR-1a* expression, especially at 4 days after inoculation (Fig. 6). However, in powdery mildew-infected NahG plants GSH and OTC treatments did not induce *PR-1a* expression during this early stage of pathogenesis at any concentrations tested (2 and 4 mM), suggesting that *PR-1a* is not directly involved in the defence responses induced by these treatments.

DISCUSSION

We found that SA-deficient NahG tobacco is significantly more susceptible to the powdery mildew fungus *E. longipes* as compared to wild-type control cv. Xanthi plants. It is known that SA is a key component of plant defence responses to biotrophic pathogens (Malamy *et al.* 1990; Gaffney *et al.* 1993; Delaney *et al.* 1994; Bari & Jones 2009; Vlot *et al.*, 2009; Klessig *et al.* 2018; Han *et al.* 2019). For example, SA-deficient *A. thaliana* NahG plants display enhanced susceptibility to the powdery mildew *Erysiphe* (syn. *Golovinomyces*) *orontii*, as judged by faster conidial development and more severe symptoms (Reuber *et al.* 1998). Since *nahG* encodes a salicylate hydroxylase that converts SA to catechol, it could be possible that, besides SA-deficiency, catechol accumulation also contributes to the enhanced susceptibility of NahG tobacco to *E. longipes*. Indeed, treatment of wild-type *Arabidopsis* with catechol compromised resistance to bacteria, and application of catalase to NahG or catechol-treated wild-type plants partially restored resistance, suggesting that the deleterious effect of catechol could result from compromised plant antioxidant capacity (van Wees & Glazebrook 2003). In fact, it is known that in NahG tobacco, antioxidants, *e.g.* catalase and GSH, are down-regulated and this is associated with a compromised virus resistance (Király *et al.* 2002). Also, *Arabidopsis* NahG plants contain reduced levels of the phytoalexin camalexin (Heck *et al.*, 2003) and some *pad* (phytoalexin/camalexin/-deficient) mutants, *e.g. pad2*, have a NahG-like phenotype (low levels of SA, enhanced

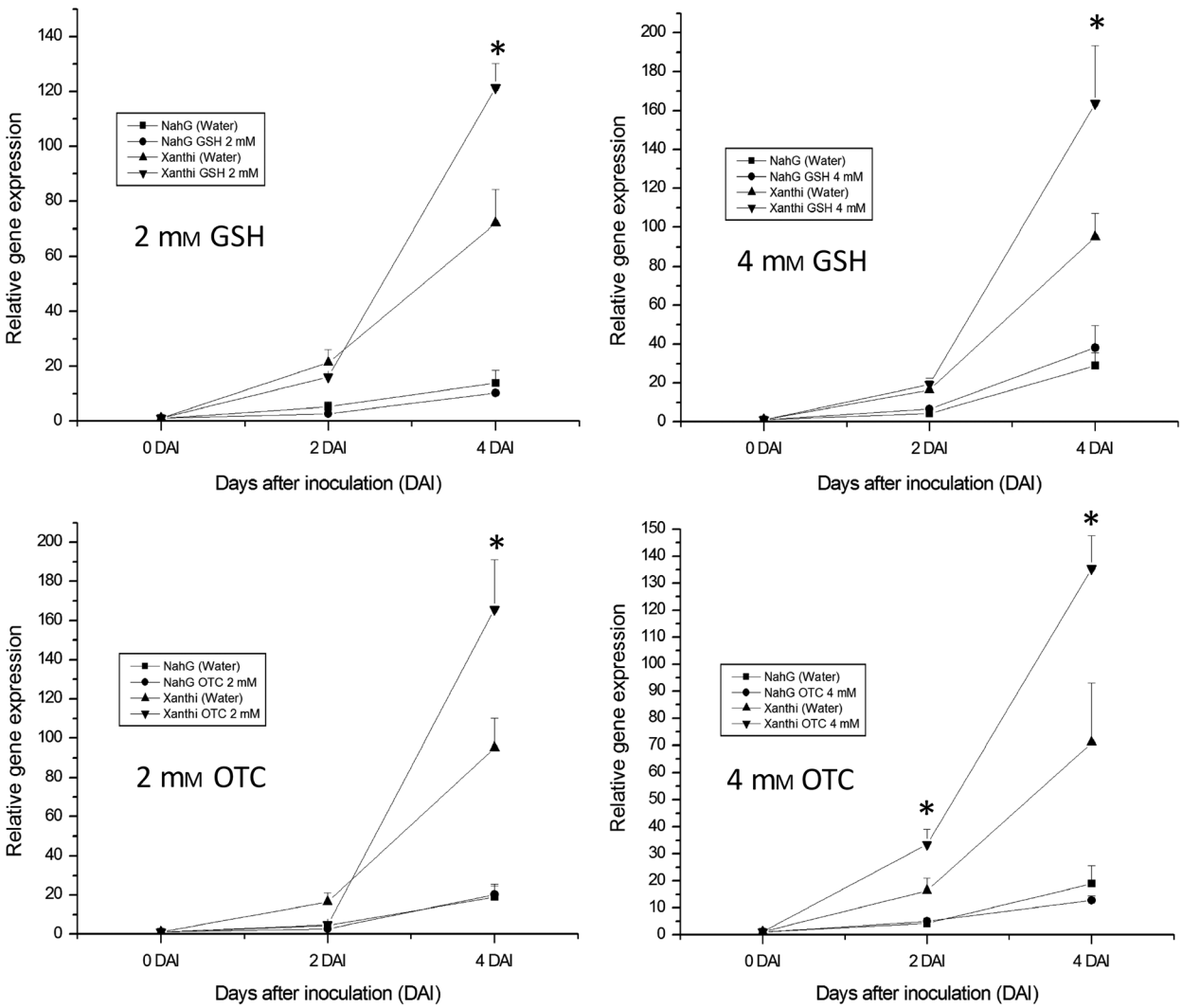

Fig. 6. Expression of the tobacco pathogenesis-related 1-a gene (*PR-1a*) as detected by RT-qPCR in wild-type *Nicotiana tabacum* cv. Xanthi and SA-deficient *N. tabacum* cv. Xanthi *NahG* tobacco infected with *Euoidium longipes* 2 and 4 days after inoculation (DAI). Right leaf halves were infiltrated with 2 and 4 mM reduced glutathione (GSH) or R-2-oxothiazolidine-4-carboxylic acid (OTC), left leaf halves with pH 6.8 tap water 3 h before inoculation. Asterisks (*) indicate statistically significant differences (Student's *t*-test, $P = 0.05$) between control (water) and GSH/OTC-treated leaf halves of the respective plant genotype.

susceptibility). Remarkably, *Pad2* encodes GSH1, the enzyme catalysing GSH production, suggesting that catechol may cause disease susceptibility by inhibiting GSH biosynthesis (see in Frendo *et al.* 2013 and references within). On the other hand, the Ni-hyperaccumulator *Thlaspi goesingense* contains elevated levels of both SA and catechol and displays enhanced sensitivity to powdery mildew (*Erysiphe cruciferarum*), failing to induce SA biosynthesis after infection (Freeman *et al.* 2005). This implies that – at least in certain plant–pathogen interactions – SA levels may be more important than catechol in determining resistance *versus* susceptibility. The above-mentioned data suggest that catechol may or may not play a role in conferring enhanced pathogen susceptibility in SA-deficient NahG plants, depending on the particular host–pathogen combination. However, the role of SA in resistance of tobacco to two different species of powdery mildew fungus has been demonstrated. Achuo *et al.* (2004) found that activating the SA-mediated pathway with a synthetic SA analogue (benzothiadiazole) induced penetration resistance to *Oidium neolycopersici* in wild-type tobacco. Furthermore, infection of SA-deficient NahG tobacco with *O. neolycopersici* and *G. orontii* resulted in a higher disease index and enhanced symptom development (Achuo *et al.* 2004; Gullner *et al.* 2017a). Here, we demonstrate that SA-deficient NahG tobacco displays enhanced susceptibility to another powdery mildew fungus, *E. longipes*, manifested as an increase not only in symptom severity but also in qPCR-assayed pathogen accumulation (Figs 1 and 3).

Our results also show that in SA-deficient NahG tobacco enhancement of GSH contents by treatments with GSH or the synthetic cysteine and GSH precursor OTC may counterbalance the lack of SA and restore normal levels of susceptibility to *E. longipes* found in wild-type plants. The pivotal role of GSH in plant disease resistance has been reported for several viral, bacterial and fungal pathogens, including powdery mildews (Fodor *et al.* 1997; Vanacker *et al.* 1998; Foyer & Rennenberg 2000; Kuźniak & Skłodowska 2004a, b; Zechmann *et al.* 2005; Höller *et al.* 2010; Ghanta *et al.* 2011; Király *et al.* 2012;

Harrach *et al.* 2013; De *et al.* 2018). In particular, high levels of apoplastic glutathione are associated with resistance of *e.g.* barley to the powdery mildew *Blumeria graminis* f. sp. *hordei* (Vanacker *et al.* 1998), a similar effect to that observed in our study in tobacco infiltrated with glutathione or OTC and infected with the powdery mildew *E. longipes*. Furthermore, in transgenic tobacco plants with high glutathione contents, an increased resistance to *Pseudomonas syringae* pv. *tabaci* is coupled to elevated levels of SA (Ghanta *et al.* 2011), and exogenous treatment with the natural nitric oxide (NO) donor S-nitrosoglutathione (GSNO) results in elevated GSH concentrations required for SA accumulation and resistance of *A. thaliana* to *P. syringae* pv. *tomato* (Kovacs *et al.* 2015). On the other hand, genetic inhibition of glutathione accumulation in *A. thaliana* leads to decreased SA levels (Han *et al.* 2013). These results imply that GSH is a central regulator of SA-dependent pathogen defence in plants. However, GSH may also induce plant disease resistance independently of SA. *A. thaliana* carrying the GSH-deficient mutation *phytoalexin-deficient 2-1* (*pad 2-1*) displays ca. 20% of GSH levels and lower SA accumulation than found in wild-type plants (Dubreuil-Maurizi & Poinssot, 2012) and enhanced susceptibility to different pathogens (Glazebrook & Ausubel 2004; Parisy *et al.* 2007; Schlaeppi *et al.* 2010). Interestingly, the pad 2-1 mutant was hyper-susceptible to the oomycete *Phytopthora porri*, and artificial elevation of SA levels did not elicit any resistance to *P. porri*, suggesting that, at least in this particular plant–pathogen interaction, GSH can induce disease resistance independently of SA (Roetschi *et al.* 2001). The present study demonstrates a similar phenomenon in NahG tobacco, where elevated levels of GSH are capable of enhancing basal resistance (*i.e.* restoring normal levels of susceptibility) to the powdery mildew *E. longipes* in the absence of SA accumulation.

Our finding that GSH may induce resistance to *E. longipes* independently of SA is supported by the negligible levels of expression of an SA-mediated pathogenesis-related gene (*NtPR-1a*) in powdery mildew-infected, GSH- or OTC-treated NahG tobacco, as compared to wild-type plants (Figs 1 and 6). The up-regulation of *PR-1a* is known to be a reliable marker of the SA-mediated defence pathway (Malamy *et al.,* 1990; Delaney *et al.,* 1994; Durrant & Dong 2004; Van Loon *et al.* 2006; Breen *et al.* 2017). Transcript analysis revealed that both transient and constitutive elevation of *in planta* glutathione levels by 'GSH feeding' or overexpression of γ-glutamylcysteine synthetase (γ-ECS, a key enzyme of GSH biosynthesis), respectively, leads to enhanced *PR-1a* gene expression (Gomez *et al.* 2004; Ghanta *et al.* 2011, 2014). Besides an increase in *PR-1a* expression, transgenic plants overexpressing γ-ECS display elevated levels of SA and enhanced resistance to *P. syringae* pv. *tabaci* (Ghanta *et al.* 2011). The fact that GSH/OTC treatments induce resistance to *E. longipes* in SA-deficient NahG plants without any induction of *PR-1a* expression suggests that *PR-1a* is not directly involved in the defence responses induced by elevated levels of glutathione.

We found that GSH treatments, as compared to OTC treatments, increase the ratio of oxidised glutathione (GSSG) to total glutathione in both SA-deficient and wild-type tobacco infected with *E. longipes*, but GSSG accumulation is much more significant in GSH-treated and powdery mildew-infected SA-deficient NahG plants (Fig. 4, Figure S1). GSSG is reduced by glutathione reductase (GR) to replenish the GSH pool, and GR activity and expression of the coding genes are activated by SA (Fodor *et al.* 1997; Király *et al.* 2002; Mhamdi *et al.* 2010). Also, it has been shown that the antioxidant capacity of NahG tobacco is down-regulated and the GSSG/GSH ratio dramatically increased within the first 4 days after inoculation with *Tobacco mosaic virus* (TMV), concomitant with enhanced susceptibility, as compared to wild-type plants (Király *et al.* 2002). This suggests that an impaired SA-mediated defence to biotrophic pathogens may confer a loss of the plant ability to regenerate reduced glutathione (GSH), a possible cause of susceptibility of *e.g.* tomato to the bacterial pathogen *P. syringae* pv. *tomato*. Decreasing GSH contents and the accumulation of GSSG were observed in an inoculated susceptible cultivar, while the maintenance of GSH pool homeostasis, including regulation of GSSG levels, seems to contribute to resistance to this bacterium (Kuźniak & Skłodowska 2004b). Our results demonstrate that in SA-deficient NahG tobacco a massive, 50–200% increase in glutathione levels may confer enhanced basal resistance to the powdery mildew *E. longipes* even if GSSG accumulation is higher than in wild-type plants. In fact, the high levels of GSSG in GSH- or OTC-infiltrated NahG tobacco could indicate the pivotal role of glutathione in restoring normal susceptibility in the background of SA deficiency. Importantly, the initially high ratio of GSSG to total glutathione gradually decreases during the first 14 days of pathogenesis. This is in line with the observation that an initial transient increase in GSSG levels is required for the activation of defence signalling in response to *e.g.* biotrophic pathogens like powdery mildews (Vanacker *et al.* 2000; Kovacs *et al.* 2015).

Taken together, we have shown that SA-deficient NahG tobacco is hyper-susceptible to *E. longipes*, displaying severe powdery mildew symptoms and increased pathogen accumulation as compared to wild-type plants. Importantly, artificial elevation of glutathione levels in SA-deficient NahG tobacco through treatment with GSH or the synthetic cysteine and GSH precursor OTC restored susceptibility to *E. longipes* to the extent seen in wild-type plants (*i.e.* enhanced basal resistance). Expression of the SA-mediated pathogenesis-related gene (*NtPR-1a*) did not increase significantly in powdery mildew-infected GSH- or OTC-pretreated NahG tobacco, suggesting that this *PR* gene may not be directly involved in the defence responses induced by GSH. Our results demonstrate that artificial elevation of glutathione can significantly reduce susceptibility to powdery mildew in a salicylic acid-deficient tobacco. Further research should elucidate the exact mechanisms of how glutathione induces resistance to powdery mildew infections in crop plants.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Ratio of oxidised glutathione (GSSG) to total glutathione (GSH + GSSG) in SA-deficient *Nicotiana tabacum* cv. Xanthi *NahG* and wild-type control *N. tabacum* cv. Xanthi tobacco during *Euoidium longipes* infection in leaf halves infiltrated with 2 and 4 mM reduced glutathione (GSH) or R-2-oxothiazolidine-4-carboxylic acid (OTC) 3 h before inoculation (based on data presented in Figs 4 and 5). DAI: days after inoculation. Asterisks (*) indicate statistically significant differences between tobacco genotypes. For pair-wise analysis Student's *t*-test was used ($P = 0.05$).

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