Glutathione contributes to resistance responses to TMV through a differential modulation of salicylic acid and reactive oxygen species

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Abstract
Systemic acquired resistance (SAR) is induced by pathogens and confers protection against a broad range of pathogens. Several SAR signals have been characterized, but the nature of the other unknown signalling by small metabolites in SAR remains unclear. Glutathione (GSH) has long been implicated in the defence reaction against biotic stress. However, the mechanism that GSH increases plant tolerance against virus infection is not entirely known. Here, a combination of a chemical, virus-induced gene-silencing-based genetics approach, and transgenic technology was undertaken to investigate the role of GSH in plant viral resistance in Nicotiana benthamiana. Tobacco mosaic virus (TMV) infection results in increasing the expression of GSH biosynthesis genes NbECS and NbGS, and GSH content. Silencing of NbECS or NbGS accelerated oxidative damage, increased accumulation of reactive oxygen species (ROS), compromised plant resistance to TMV, and suppressed the salicylic acid (SA)-mediated signalling pathway. Application of GSH or L-2-oxothiazolidine-4-carboxylic acid (a GSH activator) alleviated oxidative damage, decreased accumulation of ROS, elevated plant local and systemic resistance, enhanced the SA-mediated signalling pathway, and increased the expression of ROS scavenging-related genes. However, treatment with buthionine sulfoximine (a GSH inhibitor) accelerated oxidative damage, elevated ROS accumulation, compromised plant systemic resistance, suppressed the SA-mediated signalling pathway, and reduced the expression of ROS-regulating genes. Overexpression of NbECS reduced oxidative damage, decreased accumulation of ROS, increased resistance to TMV, activated the SA-mediated signalling pathway, and increased the expression of the ROS scavenging-related genes. We present molecular evidence suggesting GSH is essential for both local and systemic resistance of N. benthamiana to TMV through a differential modulation of SA and ROS.

KEYWORDS
glutathione, Nicotiana benthamiana, reactive oxygen species, salicylic acid, systemic resistance, tobacco mosaic virus
1 | INTRODUCTION

The tripeptide thiol glutathione (GSH: \( \gamma \)-glutamyl-\( \gamma \)-cysteinyl-glycine) is widely distributed in eukaryotes and appears to be essential in plant cells. GSH is synthesized in two steps catalysed by two adenosine triphosphate (ATP)-dependent enzymes, \( \gamma \)-glutamylcysteine synthetase (GSH1, also referred to as \( \gamma \)-ECS) and glutathione synthetase (GSH2, also referred to as GSHS), from its constituent amino acids (cysteine, glutamic acid, and glycine) (Noctor et al., 2002). GSH has many important functions in plants, including protection against oxidative stress, detoxification of heavy metals and electrophilic xenobiotics, redox control, long-distance transport of reduced sulphur, ascorbate-glutathione cycle as a nonenzymatic antioxidant, serving as electron donor for biochemical reactions, plant growth and development, and stress defence gene expression (Ball et al., 2004; Foyer et al., 1997; Ghanta et al., 2011; Gullner & Kőmivés, 2006; Ishikawa et al., 2010; Kocsy et al., 2013; Künstler et al., 2019; May et al., 1998; Mhamdi et al., 2010; Noctor et al., 2012; Pasternak et al., 2008; Vernoux et al., 2000). An increasing number of studies indicate that GSH plays important roles in tolerance of abiotic stresses such as drought, heavy metal, chilling, salt, high light, and frost (Cheng et al., 2015; Gomez et al., 2004; Kocsy et al., 2000; Kumar et al., 2009; Ruiz & Blumwald, 2002; Zechmann, 2014). In addition, GSH has been implicated in defence reactions against biotic stress, including viral, fungal, and bacterial infections (De et al., 2018; Farkas et al., 1960; Fodor et al., 1997; Király et al., 2002; Künstler et al., 2019; Mukaihara et al., 2016; Noctor et al., 2012; Zechmann, 2014, 2020). The significant role of GSH in the regulation of defence responses against pathogens has been recognized for a long time due to its antioxidant function (Fodor et al., 1997; Ghanta & Chattopadhyay, 2011; Gullner & Kőmivés, 2001; Gullner et al., 2017; Király et al., 2002; Noctor et al., 2012; Zechmann, 2020). For example, the GSH levels and its redox state are related to the response to infection by the bacterial pathogen Pseudomonas syringae in two different tomato cultivars (Kuźniak & Sklodowska, 2004). However, an increasing amount of research has indicated that the role of GSH-mediated plant disease resistance against pathogens is not dependent on the antioxidant function of GSH, but GSH status can regulate several important signals that lead to activation of defence responses during plant biotic stress (Feechan et al., 2005; Ghanta et al., 2011a; Han et al., 2013; Kovacs et al., 2015; Zhang et al., 2020). Small-molecule phytohormones, such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), gibberellins (GAs), cytokinins (CKs), abscisic acid (ABA), and brassinosteroids (BRs), are known to play pivotal roles in mediating the plant defence signalling network against pathogens (Berens et al., 2017; Berger et al., 2020; Lu & Yao, 2018). Recent studies indicate that GSH modulates the defence signalling network by cross-communication with phytohormones during biotic stress (Ghanta et al., 2011, 2014; Gullner et al., 2017; Han et al., 2013; Künstler et al., 2019, 2020; Mhamdi et al., 2013). Overexpression of the Solanum lycopersicum \( \gamma \)-glutamylcysteine synthetase (LeECS) gene in transgenic Nicotiana tabacum plants significantly increases resistance against the biotrophic bacterial pathogen Pseudomonas syringae pv. tabaci. GSH confers resistance against biotrophic infection, probably through the NPR1-dependent SA-mediated pathway (Ghanta et al., 2011). Viral infection results in increasing GSH levels in plant cells and elevated endogenous GSH levels improve virus resistance. A tolerant pumpkin species showed a much stronger accumulation of GSH and attenuated disease symptoms during zucchini yellow mosaic virus (ZYMV) infection at 14 days postinoculation (dpi). However, the susceptible species showed much lower GSH accumulation and developed stronger symptoms (Zechmann et al., 2005, 2006; Zecharia & Müller, 2008). Several lines of evidence have suggested that the artificial increase of cellular GSH content in plant tissues by application of GSH or the synthetic cysteine precursor L-2-oxothiazolidine-4-carboxylic acid (OTC) can significantly improve the disease resistance of plants (Gullner et al., 2017; Künstler et al., 2019, 2020; Zechmann et al., 2007b). OTC pretreatment of tobacco or pumpkin plants significantly decreased tobacco mosaic virus (TMV) and ZYMV accumulation and also attenuated disease symptoms (Gullner et al., 1999; Zechmann et al., 2007b). OTC treatment also alleviated viral disease symptoms, although they did not markedly decrease viral accumulation in plum pox virus-infected pea and peach tissues (Clemente-Moreno et al., 2010, 2012, 2013). The mechanism by which GSH increases plant tolerance against viral infection is not entirely known, although GSH and SA are interconnected in conferring resistance to TMV in tobacco (N. tabacum) and constitutive GSH synthesis is required for controlling potato virus X (PVX) accumulation in Nicotiana benthamiana (De et al., 2018; Fodor et al., 1997; Király et al., 2002; Künstler et al., 2019).

Reactive oxygen species (ROS) are produced as by-products of aerobic metabolism in plants and were initially recognized as toxic by-products that cause oxidative damage to DNA, proteins, and lipids (Mittler, 2017). In recent years, more and more studies have focused on the role ROS play as important signalling molecules in plants, regulating a broad range of processes such as growth, development, and especially responses to biotic and abiotic environmental stresses (Baxter et al., 2014; Bechtold et al., 2013; Das & Roychoudhury, 2014; Mignolet-Sprüyt et al., 2016; Pöör, 2020; Radwan et al., 2010; Sharma et al., 2012; Shi et al., 2014; Vuleta et al., 2016; Xu et al., 2019). ROS have a dual role in infected plants, as high concentrations of ROS promote programmed cell death (PCD) of infected plant cells and death/limitation of invading pathogens, while low ROS concentrations function in signalling of host defence responses in healthy plant cells adjacent to infection sites (Dat et al., 2000; Levine et al., 1994; Pogány et al., 2009; Torres, 2010; Torres et al., 2005). Accordingly, plants often suffer from oxidative stress (also called oxidative burst) during infection by pathogenic microorganisms, that is, the rapid and massive accumulation of ROS in infected tissues. To avoid the excessive accumulation of ROS in plant cells, plants have developed complicated scavenging and regulation pathways to monitor ROS redox homeostasis (Das & Roychoudhury, 2014; Foyer & Noctor, 2009;
Podgórska et al., 2017). Redox homeostasis of plants during stressful conditions is maintained by antioxidative systems composed of nonenzymatic and enzymatic antioxidants. The nonenzymatic low molecular compounds include ascorbic acid (ASC), reduced glutathione (GSH), α-tocopherol, flavonoids, carotenoids, phenolics, and proline (Das & Roychoudhury, 2014; De Gara et al., 2003; Hernández et al., 2016). Catalase (CAT), glutathione-S-transferase (GST), superoxide dismutase (SOD), guaiacol peroxidase (GPX) and enzymes related to the ascorbate-glutathione cycle, such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), glutathione peroxidase (GP), and dehydroascorbate reductase (DHAR), serve as potent enzymatic antioxidants (Das & Roychoudhury, 2014; Gill & Tuteja, 2000). N. benthamiana. Evidence is provided that GSH is a crucial regulator in local and systemic resistance responses against TMV infection and has great potential in engineering crops with enhanced resistance against pathogen infections.

2 | RESULTS

2.1 | Identification of NbECS and NbGS

The complete open reading frames (ORFs) of NbECS and NbGS were cloned from N. benthamiana. We successfully obtained two approximately 1569-bp and 1668-bp fragments of NbECS and NbGS genes, respectively (Figure S1). The obtained fragments were purified and cloned into the pMD19-T vector, and the recombinant plasmid was confirmed by plasmid PCR (data not shown). The obtained fragments were sequenced. Nucleotide BLAST analysis revealed that the nucleotide sequences of NbECS and NbGS shared 100% and 96% coverage, and 92.05% and 88.38% nucleotide identities with S. lycopersicum GSH1 (SIGSH1) and SIGSH2 sequences available in GenBank (accessions AF017983 and AF017984, respectively).

2.2 | GSH is induced by TMV infection

To investigate whether GSH is involved in the response to viral infection, we examined the expression of NbECS and NbGS and the GSH content in N. benthamiana plants infected with green fluorescent protein (GFP)-tagged TMV, a recombinant TMV that is infectious. The results showed that the expression of NbECS and NbGS was rapidly induced in inoculated leaves at 12 and 24 hours postinoculation (hpi) (Figure 1a,b). In addition, the GSH content was increased in inoculated leaves at 12 hpi (Figure 1c). The GSH content was also significantly enhanced in systemic leaves (upper noninoculated leaves) at 5 dpi (Figure 1d).

2.3 | Silencing of NbECS or NbGS compromises plant resistance to TMV in N. benthamiana plants

Next, a tobacco rattle virus (TRV)-based VIGS system was used to suppress the NbECS or NbGS mRNA levels in N. benthamiana plants (Zhu et al., 2014). No obvious difference of the phenotype was observed between NbECS- or NbGS-silenced plants and control plants (TRV:00) (Figure S2a). To investigate the gene silencing efficiency of NbECS and NbGS genes, quantitative reverse transcription PCR (RT-qPCR) was used to examine the expression levels of NbECS and NbGS in 12 days after silencing plants by VIGS using primers that anneal to NbECS and NbGS outside the region targeted for silencing (Wangdi et al., 2010; Zhu et al., 2019). The results showed that the transcript levels of NbECS and NbGS were significantly reduced in the NbECS- or NbGS-silenced plants as compared to the control plants (Figure S2b,c). The GSH content was also measured in NbECS- or NbGS-silenced plants. Our results indicated that the GSH content in the NbECS- or NbGS-silenced plants was lower than in the control plants (Figure S2d).

Our results indicated that there was a significant increase in the number of GFP fluorescent foci in NbECS- or NbGS-silenced plants as compared to control plants (Figure 1e,f). The conclusion is consistent with RT-qPCR and western blotting analysis of viral accumulation. RT-qPCR results showed that the TMV RNA levels in the leaves of the NbECS- or NbGS-silenced plants were higher than in control plants (Figure 1g). Western blotting analysis indicated that the coat protein levels of TMV (TMV-CP) were significantly increased in NbECS- or NbGS-silenced plants in comparison with control plants (Figure 1h). We also monitored viral replication and spread in the noninoculated upper leaves at 6 dpi by direct observation of the number of GFP fluorescent foci and RT-qPCR analysis of TMV RNA levels (Figure S2e–g). GFP fluorescence imaging (Figure S2e), GFP fluorescent foci quantification analysis (Figure S2f), and RT-qPCR results (Figure S2g) confirmed that increased viral accumulation was observed in upper noninoculated leaves of NbECS- or NbGS-silenced plants as compared to control plants. Therefore, the suppression of NbECS or NbGS compromises plant resistance to TMV.

2.4 | Silencing of NbECS or NbGS increases TMV-induced oxidative damage and the accumulation of ROS

The cytomembrane could be adversely affected by oxidative damage induced by pathogen infection. Electrolyte leakage and malondialdehyde (MDA) content are indicators of membrane lipid peroxidation, cell death, and cytomembrane penetrability (Zhang et al., 2012). Therefore, MDA content and electrolyte leakage were investigated in NbECS- or NbGS-silenced plants (inoculated leaves) at 3 days after TMV-GFP infection (Figure 2a,b). No obvious difference of MDA content and electrolyte leakage was detected between NbECS- or NbGS-silenced plants and control plants (TRV:00) without TMV-GFP.
infection (Figure 2a,b). However, the MDA content was significantly increased by TMV-GFP infection in NbECS- or NbGS-silenced plants and control plants (TRV:00) (Figure 2a). NbECS- or NbGS-silenced plants had more MDA formation than control plants (TRV:00) after TMV-GFP infection, indicating that silencing of NbECS or NbGS accelerated peroxidation of membrane lipids during TMV-GFP infection (Figure 2a). Consistent with the MDA contents, the level of leakage was also significantly increased in NbECS- or NbGS-silenced plants as compared to control plants (TRV:00) after TMV-GFP inoculation, implying that cytomembranes of NbECS- or NbGS-silenced plants suffered more oxidative damage during TMV-GFP infection (Figure 2b).

The prolonged production of ROS in high concentrations is correlated with plant cell death and increased susceptibility to pathogens infection (Mittler et al., 2004; Overmyer et al., 2003; Zhu et al., 2015). Therefore, we first determined the levels of $O_2^-$ and $H_2O_2$ by nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining in NbECS- or NbGS-silenced and control (TRV:00) plants after TMV-GFP infection at 3 dpi. Asterisks represent significant difference determined by Student’s t test (**p < 0.001). (g) RT-qPCR analysis of TMV replication levels in NbECS- or NbGS-silenced and control (TRV:00) plants after TMV-GFP infection at 3 dpi. Bars represent mean and standard deviation of values obtained from three biological replicates. Different lower case letters indicate significant differences (p < 0.05). (h) Western blotting analysis of coat protein accumulation of TMV in NbECS- or NbGS-silenced and control (TRV:00) plants after TMV-GFP infection at 3 dpi. RuBisCO proteins were used as loading controls and were stained by Ponceau S.
FIGURE 2  Silencing of NbECS or NbGS accelerated tobacco mosaic virus (TMV)-induced oxidative damage in Nicotiana benthamiana plants and suppresses the salicylic acid (SA)-mediated defence signalling pathway. Malondialdehyde (MDA) content (a) and electrolyte leakage (b) were measured in leaves of NbECS- or NbGS-silenced plants after TMV-GFP infection at 3 days postinoculation (dpi). (c) The levels of $O_2^{•−}$ and $H_2O_2$ were determined by nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining of NbECS- or NbGS-silenced leaves after TMV-GFP infection (3 dpi). (d) $O_2^{•−}$ content was examined by the hydroxylamine oxygenation reaction method. (e) $H_2O_2$ content was measured by using a sensitive quantitative Amplex red hydrogen peroxide/peroxidase assay kit. TRV:00, nonsilenced plants inoculated with 0.02 M phosphate-buffered saline (PBS); TRV:NbECS, NbECS-silenced plants inoculated with 0.02 M PBS; TRV:NbGS, NbGS-silenced plants inoculated with 0.02 M PBS; TRV:00+TMV-GFP, nonsilenced plants inoculated with TMV-GFP at 3 dpi; TRV:NbECS+TMV-GFP, NbECS-silenced plants inoculated with TMV-GFP at 3 dpi; TRV:NbGS+TMV-GFP, NbGS-silenced plants inoculated with TMV-GFP at 3 dpi. (f) Analysis of salicylic acid (SA) levels in NbECS- or NbGS-silenced plants. (g) Quantitative reverse transcription PCR (RT-qPCR) analysis of the expression of SA biosynthesis gene NbICS1 in NbECS- or NbGS-silenced plants. (h) RT-qPCR analysis of the expression of SA signalling pathway gene NbNPR1 in NbECS- or NbGS-silenced plants. (i–k) RT-qPCR analysis of the expression of defence-related genes associated with the SA-mediated defence pathway in NbECS- or NbGS-silenced plants. Bars represent mean and standard deviation of values obtained from three biological replicates. Significant differences ($p < 0.05$) are denoted by different lower case letters.
stained areas in NbECS- or NbGS-silenced plants were much larger and darker than in control plants after TMV-GFP infection, indicating that NbECS- or NbGS-silenced plants accumulated more ROS (Figure 2c). To determine the H$_2$O$_2$ and O$_2^-$ contents more precisely, we measured the H$_2$O$_2$ content by a sensitive quantitative Amplex red hydrogen peroxide/peroxidase assay kit, and the hydroxylamine oxygenation reaction method was used to detect the O$_2^-$ content. Our results showed that the O$_2^-$ and H$_2$O$_2$ contents were significantly increased in NbECS- or NbGS-silenced plants (inoculated leaves) as compared to control plants (TRV:00) at 3 days after TMV-GFP inoculation (Figure 2d,e).

### 2.5 Silencing of NbECS or NbGS suppresses SA-mediated signalling pathway

To further investigate the probable mechanisms of action of GSH in plant systemic resistance against TMV, we determined phytohormone SA content, and the expression of SA biosynthetic, signalling and defence marker genes in NbECS- or NbGS-silenced plants. The endogenous levels of SA were significantly reduced in NbECS- or NbGS-silenced plants compared with control plants (TRV:00) (Figure 2f). RT-qPCR results showed that the expression of NbICS1 (SA biosynthetic gene) and NbNPR1 (SA signalling gene) was markedly decreased in NbECS- or NbGS-silenced plants as compared to control plants (TRV:00) (Figure 2g,h). SA-mediated defence genes play a significant role in plant defence responses (Zhu et al., 2014). The transcript levels of NbPR1, NbPR2, and NbPR5 (SA defence marker genes) were significantly reduced in NbECS- or NbGS-silenced plants as compared to control plants (Figure 2i–k).

### 2.6 Application of GSH, OTC, and buthionine sulfoximine alter plant systemic resistance to TMV

Several lines of evidence suggested that the artificial increase of cellular GSH content in plant tissues by application of GSH or the synthetic cysteine precursor OTC can significantly improve the disease resistance of plants (Gullner et al., 2017; Künstler et al., 2019, 2020; Zechmann et al., 2007b). The functions of GSH in plants have also been studied using buthionine sulfoximine (BSO), which is considered to be a specific inhibitor of GSH synthesis (Gullner & Dodge, 2000; Koprivova et al., 2010; Marquez-Garcia et al., 2014). To further investigate the function of GSH in plant systemic resistance against TMV, *Nicotiana benthamiana* plants were pretreated on their primary (local) leaves with water, various concentrations of GSH, OTC, or BSO, and then inoculated with TMV-GFP on the upper leaves (Figure S3a). No obvious difference of the phenotype was observed between GSH-, OTC-, or BSO-treated plants and control plants (water treatment) (Figure S3b). The GSH content was also examined in GSH-, OTC-, or BSO-treated plants. The results indicated that the GSH content was elevated in the GSH- or OTC-treated plants to some extent and more or less decreased in BSO-treated plants as compared to water-treated plants (control) (Figure S3c).

Our results showed that there was a marked reduction in the number of GFP fluorescent foci in inoculated leaves of local 10 mM GSH or OTC (1 and 10 mM) treatment by spraying, as compared to water-treated plants at 3 dpi after TMV-GFP infection (Figure 3a,b). However, the number of GFP fluorescent foci significantly increased in inoculated leaves of 0.1 and 1 mM BSO treatments as compared to control plants (water treatment) (Figure 3a,b). RT-qPCR results showed that the TMV RNA levels were reduced in the leaves of the 10 mM GSH- or OTC-treated plants to some extent as compared to control plants at 3 dpi after TMV-GFP infection (Figure 3c). Nevertheless, elevated TMV transcript levels were detected in inoculated leaves of 0.1 and 1 mM BSO treatments as compared to control plants (water treatment) (Figure 3c). The coat protein levels of TMV (TMV-CP) were significantly reduced in the leaves of the 10 mM GSH or OTC (1 and 10 mM)-treated plants and enhanced in 0.1 and 1 mM BSO-treated plants in comparison to control plants at 3 dpi after TMV-GFP infection (Figure 3d). We further monitored TMV replication and spread in the systemic upper leaves at 6 dpi. GFP fluorescence imaging (Figure 3e), GFP fluorescent foci quantification analysis (Figure 3f), and RT-qPCR results (Figure 3g) confirmed that elevated viral accumulation was observed in upper noninoculated leaves of 0.1 and 1 mM BSO-treated plants compared
with water-treated plants. Furthermore, reduced viral accumulation was detected in upper systemic leaves of GSH- or OTC-treated plants compared with control plants (Figure 3e–g). Taken together, these results suggest that application of GSH or OTC to *N. benthamiana* plants elevates, while treatment with BSO compromises, plant local and systemic resistance to TMV infection.

2.7 Application of GSH, OTC, and BSO altered oxidative damage and the accumulation of ROS under TMV-GFP infection

No obvious differences in MDA contents and electrolyte leakage were found between GSH-, OTC-, or BSO-treated plants and
water-treated plants without TMV-GFP infection (Figure S4a,b). However, the MDA contents were significantly decreased in 10 mM GSH- and 1 and 10 mM OTC-treated plants, and increased in 0.1 and 1 mM BSO-treated plants in comparison to control plants at 3 dpi after TMV-GFP infection (Figure 4a). The level of leakage was also significantly reduced in 10 mM GSH- and 1 and 10 mM OTC-treated plants and enhanced in 0.1 and 1 mM BSO-treated plants in comparison to control plants at 3 dpi after TMV-GFP infection (Figure 4b). These results suggest that application of BSO alleviates TMV-induced oxidative damage and treatment with GSH or OTC alleviates oxidative damage during TMV infection.

We also examined the levels of ROS in GSH-, OTC-, or BSO-treated plant leaves at 3 dpi after TMV-GFP infection. Areas of slight staining by NBT and DAB occurred in GSH-, OTC-, or BSO-treated plants and water-treated plants without TMV-GFP infection (Figure 4c). NBT and DAB staining results showed that the $O_2^-$ and $H_2O_2$ contents were significantly enhanced in GSH-, OTC-, or BSO-treated plants and control plants during TMV-GFP infection (Figure 4c). However, GSH- or OTC-treated plants exhibited fewer staining areas than control plants after TMV-GFP infection, indicating that GSH- or OTC-treated plants accumulated less ROS (Figure 4c). However, more staining areas were observed in BSO-treated plants than control plants at 3 dpi during TMV-GFP infection, suggesting that BSO-treated plants accumulated more ROS (Figure 4c). Next, the levels of ROS were measured precisely. No obvious differences in $O_2^-$ and $H_2O_2$ levels were detected between GSH-, OTC-, or BSO-treated plants and water-treated plants (control) without TMV-GFP infection (Figure S4c,d). Our results, however, showed that the $H_2O_2$ and $O_2^-$ contents were markedly reduced in GSH- or OTC-treated plants and increased in BSO-treated plants compared with water-treated plants (control) at 3 days after TMV-GFP inoculation (Figure 4d,e).

2.8 Application of GSH, OTC, and BSO altered the SA-mediated signalling pathway and the expression of ROS scavenging-related genes

To determine whether application of GSH, OTC, or BSO in N. benthamiana plants affects the SA-mediated signalling pathway, SA content, the expression of SA biosynthetic, signalling genes and SA-related defence genes were examined by high-performance liquid chromatography (HPLC) and RT-qPCR. Our results suggested that the SA content was significantly enhanced in GSH- or OTC-treated plants and reduced in BSO-treated plants compared with water-treated plants (Figure 5a). Treatment with GSH or OTC resulted in an increase in the expression of the SA biosynthetic and signalling genes NbICS1 and NbNPR1 (Figure 5b,c). However, application of BSO decreased the transcript levels of NbICS1 and NbNPR1 (Figure 5b,c). RT-qPCR indicated that the transcript levels of SA-related defence genes NbPR1, NbPR2, and NbPR5 were higher in GSH- or OTC-treated plants than in water-treated plants (Figure 5d–f). Nevertheless, the expression of NbPR1, NbPR2, and NbPR5 markedly decreased in BSO-treated plants compared with water-treated plants (Figure 5d–f).

The results related to ROS accumulation suggest that GSH may be involved in the regulation of ROS homeostasis. ROS-scavenging enzymes play essential roles in ROS cellular homeostasis under normal and stressful conditions. Increasing evidence indicates that catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX) are the three major types of ROS-scavenging enzymes (Apel & Hirt, 2004; Das & Roychoudhury, 2014; Podgórška et al., 2017). Therefore, we investigated the expression levels of 13 ROS-scavenging enzymes genes encoding CAT, SOD, or APX in the GSH-, OTC-, or BSO-treated plants using RT-qPCR. The results suggest that the expression of the six genes encoding APX (NbAPX1, NbAPX3, NbAPX4, NbAPX5, NbAPX6, and NbAPX7) were significantly increased in the GSH- or OTC-treated N. benthamiana plants compared with water-treated plants (Figure 5g, i–m). Treatment with OTC reduced the expression of NbAPX2 (Figure 5h). There were no obvious differences in the expression of NbAPX2 between the GSH- or OTC-treated N. benthamiana plants and water-treated plants (Figure 5h). Application of BSO significantly down-regulated the expression of NbAPX1, NbAPX2, NbAPX3, NbAPX5, NbAPX6, and NbAPX7 (Figure 5g–i,k–m). There were no obvious differences in the expression of NbAPX4 between the BSO-treated N. benthamiana plants and water-treated plants (Figure 5j). The three genes encoding CAT (NbCAT1, NbCAT2, and NbCAT3) were enhanced in the GSH- or OTC-treated N. benthamiana plants compared with water-treated plants (Figure 5n–p). Treatment with BSO significantly suppressed the expression of NbCAT1 and NbCAT3 (Figure 5n,p). No obvious differences of the expression of NbCAT2 were measured in the BSO-treated N. benthamiana plants and water-treated plants (Figure 5o). The expression of two genes encoding SOD (NbCu/Zn-SOD and NbFeSOD) was found to be significantly increased in the GSH- or OTC-treated N. benthamiana plants compared with water-treated plants (Figure 5q,r). The expression of NbMnSOD was also up-regulated in OTC-treated N. benthamiana plants (Figure 5s). There were no obvious differences in the expression of NbMnSOD between the GSH-treated plants and control plants (Figure 5s).
Application of BSO significantly down-regulated the expression of NbCu/Zn-SOD (Figure 5q). There were no obvious differences in the expression of NbFeSOD and NbMnSOD between the BSO-treated N. benthamiana plants and water-treated plants (Figure 5r,s). The results suggest that application of GSH or OTC may up-regulate the expression of a large number of ROS-regulating genes, while treatment with BSO may suppress the expression of a large number of ROS-regulating genes.
FIGURE 5 Application of glutathione (GSH), L-2-oxothiazolidine-4-carboxylic acid (OTC), and bithionoxime Sulfoximine (BSO) altered the salicylic acid (SA)-mediated signalling pathway and the expression of reactive oxygen species (ROS) scavenging-related genes. (a) Analysis SA levels in water-, GSH-, OTC-, or BSO-treated Nicotiana benthamiana plants. (b) Quantitative reverse transcription PCR (RT-qPCR) analysis of the expression of the SA biosynthesis gene NbICS1 in water-, GSH-, OTC-, or BSO-treated plants. (c) RT-qPCR analysis of the expression of the SA signalling pathway gene NbNPR1 in water-, GSH-, OTC-, or BSO-treated plants. (d–f) RT-qPCR analysis of the expression of defence-related genes associated with the SA-mediated defence pathway in water-, GSH-, OTC-, or BSO-treated plants. Bars represent mean and standard deviation of values obtained from three biological replicates. Significant differences (p < 0.05) are denoted by different lower case letters. (g–s) RT-qPCR analysis of the expression of catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX) genes in water-, GSH-, OTC-, or BSO-treated N. benthamiana plants. Bars represent mean and standard deviation of values obtained from three biological replicates. Asterisks represent significant differences determined by Student’s t test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

2.9 | Identification of transgenic N. benthamiana lines

The gene encoding NbECS was cloned into pCAMBIA1300 plant binary vector under the control of the CaMV 35S promoter (3SS:NbECS-GFP) (Figure S5a) and used to transform N. benthamiana leaves. Independent T<sub>1</sub> transgenic lines grown in greenhouse conditions were screened by PCR analysis. Total RNA was isolated from positive lines as well as from the nontransformed wild type (WT). First, semiquantitative RT-PCR was performed to detect the expression levels of the hygromycin gene, indicating that the levels of hygromycin gene were significantly enhanced in OE-10 and OE-18 lines (Figure S5b). However, the expression of the hygromycin gene was not detected in the nontransformed WT (Figure S5b). RT-qPCR results demonstrated that the expression levels of NbECS were significantly increased in OE-10 and OE-18 lines compared with non-transformed WT (Figure S5c). Finally, the GSH content was also measured. Our results indicated that the GSH content was significantly increased in OE-10 and OE-18 lines compared with that of WT plants (Figure S5d). Therefore, OE-10 and OE-18 lines were selected to further study the role of NbECS in resistance to TMV infection.

2.10 | Overexpression of NbECS reduced oxidative damage and the accumulation of ROS during TMV-GFP infection

Electrolyte leakage and MDA content were detected in NbECS-overexpressing plants (OE-10 and OE-18 lines) and in nontransformed WT plants during TMV-GFP infection (Figure S6a,b). No obvious difference of electrolyte leakage or MDA content occurred between the NbECS-overexpressing plants and the nontransformed WT plants in the absence of TMV-GFP infection (Figure S6a,b). However, electrolyte leakage and MDA content were significantly reduced in NbECS-overexpressing plants compared with the nontransformed WT plants after TMV-GFP infection at 3 dpi (Figure S6a,b), indicating that overexpression of NbECS in N. benthamiana plants decreased peroxidation of membrane lipids during TMV-GFP infection. Overall, these results indicate that overexpression of NbECS in N. benthamiana plants alleviates TMV-induced oxidative damage.

The levels of O<sub>2</sub>− and H<sub>2</sub>O<sub>2</sub> were determined in NbECS-overexpressing plants (OE-10 and OE-18 lines) and in nontransformed WT plants during TMV-GFP infection (Figure S6c,d). No obvious differences in the levels of O<sub>2</sub>− and H<sub>2</sub>O<sub>2</sub> were detected in NbECS-overexpressing plants and the nontransformed WT plants in the absence of TMV-GFP infection (Figure S6c,d). However, the levels of O<sub>2</sub>− and H<sub>2</sub>O<sub>2</sub> were significantly increased in NbECS-overexpressing plants and the nontransformed WT plants during TMV-GFP infection at 3 dpi (Figure S6c,d). Furthermore, the levels of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>− were significantly reduced in NbECS-overexpressing plants compared with that of WT plants after TMV-GFP infection (Figure S6c,d). Our results indicated that overexpression of NbECS in N. benthamiana plants significantly decreased the accumulation of ROS during TMV-GFP infection.

2.11 | N. benthamiana lines overexpressing NbECS showed enhanced resistance to TMV

Next, NbECS-overexpressing plants (OE-10 and OE-18 lines) were inoculated with TMV-GFP and monitored for viral replication and spread for at least 1 week. GFP fluorescence was negligible in noninoculated, NbECS-overexpressing transgenic plants. GFP fluorescence imaging (Figure 6a) and GFP fluorescent foci quantification analysis (Figure 6b) indicated that a reduced number of GFP fluorescent foci was observed in NbECS-overexpressing plants compared with WT. Furthermore, NbECS-overexpressing plants had lower TMV accumulation than WT (Figure 6c). The level of TMV-CP accumulation was significantly decreased in NbECS-overexpressing plants compared with that of WT plants (Figure 6d).

2.12 | N. benthamiana lines overexpressing NbECS enhances the SA-mediated signalling pathway and the expression of ROS scavenging-related genes

Next, we measured the SA content, the expression of SA biosynthetic, signalling genes and SA-related defence genes in N. benthamiana lines OE-10 and OE-18 overexpressing NbECS. NbECS-overexpressing plants (OE-10 and OE-18) had higher SA content than WT (Figure 6e). The expression of SA biosynthetic and signalling genes NbICS1 and NbNPR1 were significantly increased in NbECS-overexpressing plants compared with WT (Figure 6f,g). The transcript levels of SA-related defence genes NbPR1, NbPR2, and NbPR5 were also significantly increased in NbECS-overexpressing plants compared with WT (Figure 6h–j).
Next, the expression levels of 13 ROS-scavenging enzymes genes encoding CAT, SOD, or APX were measured in NbECS-overexpressing N. benthamiana lines OE-10 and OE-18 and in nontransformed WT plants. The results suggested that the expression of the six genes encoding APX, NbAPX1, NbAPX3, NbAPX4, NbAPX5, NbAPX6, and NbAPX7, were significantly increased in the OE-10 and OE-18 lines.
compared with the nontransformed WT plants (Figure 7a, c–g). There were no obvious differences in the expression of NbAPX2 between the OE-10 line and WT (Figure 7b). The three genes encoding CAT, NbCAT1, NbCAT2, and NbCAT3, were also markedly enhanced in the OE-10 and OE-18 lines compared with WT (Figure 7h–j). Furthermore, expression of the genes encoding SOD, NbCu/Zn-SOD,

**FIGURE 6** *Nicotiana benthamiana* lines overexpressing *NbECS* enhances resistance to tobacco mosaic virus (TMV) and activates the salicylic acid (SA)-mediated signalling pathway. (a) Analysis of green fluorescent protein (GFP) fluorescence in the inoculated leaves of *NbECS*-overexpressing plants (OE-10 and OE-18 lines) and nontransformed wild-type (WT) plants after TMV-GFP infection. GFP fluorescence was photographed from the inoculated leaves of *NbECS*-overexpressing plants and nontransformed WT plants after TMV-GFP infection at 3 days postinoculation (dpi). (b) Bar plot showing the ratio of GFP fluorescent area to the total area of *NbECS*-overexpressing plants and nontransformed WT plants at 3 dpi. (c) Quantitative reverse transcription PCR (RT-qPCR) analysis of TMV replication levels in the inoculated leaves of *NbECS*-overexpressing plants and nontransformed WT plants after TMV-GFP infection at 3 dpi. (d) Western blotting analysis of coat protein (CP) accumulation of TMV in the inoculated leaves of *NbECS*-overexpressing plants and nontransformed WT plants after TMV-GFP infection at 3 dpi. RuBisCO proteins were used as loading controls and were stained by Ponceau S. (e) Analysis of SA levels in *NbECS*-overexpressing plants and nontransformed WT plants. (f) RT-qPCR analysis of the expression of SA biosynthesis gene *NbICS1* in *NbECS*-overexpressing plants and nontransformed WT plants. (g) RT-qPCR analysis of the expression of SA signalling pathway gene *NbNPR1* in *NbECS*-overexpressing plants and nontransformed WT plants. (h–j) RT-qPCR analysis of the expression of defence-related genes associated with the SA-mediated defence pathway in *NbECS*-overexpressing plants and nontransformed WT plants. Bars represent mean and standard deviation of values obtained from three biological replicates. Asterisks represent significant differences determined by Student’s *t* test (*p* < 0.05, **p** < 0.01, ***p*** < 0.001)
NbFeSOD, and NbMnSOD, were significantly up-regulated in the OE-10 and OE-18 lines compared with WT (Figure 7k–m). The results suggested that NbECS may regulate the expression of a large number of ROS-associated genes.

3 | DISCUSSION

The role of GSH in plant defence and tolerance against abiotic and biotic stress has long been known (Ghanta et al., 2011a; Gullner et al., 2017; Noctor et al., 2012; Pilarska et al., 2016; Zechmann, 2014, 2020). However, how GSH regulates the plant defence signalling network against viral infection is not entirely known, although GSH and SA are interconnected in conferring resistance to TMV in tobacco and constitutive GSH synthesis is required for controlling potato virus X (PVX) accumulation in N. benthamiana (De et al., 2018; Fodor et al., 1997; Király et al., 2002; Künstler et al., 2019). Several studies have suggested the importance of GSH content and GSH-related enzymes in the physiological and biochemical responses of plants against viral infection (Hernández et al., 2016). Elevated GSH levels have often been observed in virus-infected plant cells (Hernández et al., 2017; Zechmann, 2020; Zechmann et al., 2005). The GSH content has often been associated with a resistance response to plant virus infection (De et al., 2018; Farkas et al., 1960; Fodor et al., 1997; Király et al., 2002; Künstler et al., 2019; Zechmann et al., 2007b). In this study, TMV infection induced the expression of the pathway of GSH biosynthesis genes and increased the GSH content in N. benthamiana. The pathway of GSH biosynthesis is well-established in plants and animals (Foyer & Noctor, 2011; Noctor et al., 2012). GSH is synthesized in two ATP-dependent steps through the sequential action of the enzymes γ-glutamylcysteine synthetase (γ-ECS) and GSH synthetase (GS). Knockout mutations of γ-ECS or GS result in lethal phenotypes in different eukaryotes. Knocking out the Arabidopsis expression of the GSH1 (γ-glutamylcysteine synthetase) gene causes lethality at the embryo stage (Cairns et al., 2006), whereas GSH2-deficient (GSH synthetase) lines show a seedling-lethal phenotype (Pasternak et al., 2008). VIGS has been widely used to study the function of plant genes in various biological processes (Burch-Smith et al., 2004; Senthil-Kumar & Mysore, 2014). VIGS exhibits lots of advantages compared to other functional genomics methods (Becker & Lange, 2010; Burch-Smith et al., 2004); for example, it can be used to study the function of genes where mutations are embryo-lethal or result in a severely deformed plant (Senthil-Kumar et al., 2008). Therefore, we used TRV-based VIGS to knockdown the expression of NbECS and NbGS in N. benthamiana. Silencing of NbECS or NbGS by VIGS resulted in more TMV accumulation in the inoculated leaves and noninoculated upper leaves of NbECS- or NbGS-silenced plants. These results indicate that GSH is involved in the defence of N. benthamiana in response to TMV infection.

Artificial elevation of cellular GSH contents can markedly improve the disease resistance of plants (Gullner et al., 1999; Künstler et al., 2019, 2020; Zechmann et al., 2007b). GSH content can be enhanced by treating plants with different chemicals, such as GSH, OTC, SA, or nitric oxide (NO) donor S-nitrosoglutathione (GSNO) (Gullner et al., 2017; Hartmann et al., 2004; Kovacs et al., 2015; Zechmann et al., 2007b). Treatments with OTC increases GSH content in both healthy and plum pox virus (PPV)-infected peach plantlets (Clemente-Moreno et al., 2012). Application of 1 mM OTC for 48 h in pumpkin (Cucurbita pepo) seedlings reduces the viral disease symptoms caused by ZYMV (Zechmann et al., 2007a). Similar results were also reported by Gullner et al. (1999) in N. tabacum. Treatment
fungal infections (Ghanta et al., 2011, 2014; Matern et al., 2015). In this study, overexpression of NbECS by transgenic technology in *N. benthamiana* plants markedly decreased TMV accumulation in the inoculated leaves.

The phytohormones SA, JA, and ET are known to participate in plant systemic resistance against diverse pests and pathogens (Berens et al., 2017; Berger et al., 2020; Lu & Yao, 2018). The signalling pathways influence each other through a complex network of synergistic and antagonistic interactions (Berens et al., 2017; Koornneef & Pieterse, 2008). Exogenous supply of GSH or GSNO (Kovacs et al., 2015; Künstler et al., 2019; Mateo et al., 2006) or using transgenic plants overexpressing γ-ECS (Ghanta et al., 2011, 2014) leads to markedly enhanced SA levels as well as up-regulation of expression of the defence genes. Furthermore, elevation of endogenous GSH levels in SA-deficient tobacco can compensate for SA deficiency to maintain virus (i.e., TMV) resistance (Künstler et al., 2019). Similar results were also recorded by Künstler et al. (2020), demonstrating that artificial elevation of GSH content can markedly reduce susceptibility to powdery mildew in SA-deficient tobacco. The higher level of GSH in SA-deficient plants may contribute to their alleviated viral symptoms (Wang et al., 2011). In addition, GSH plays an important functional role in the transmission of signals downstream of H$_2$O$_2$ (Han et al., 2013). Blocking GSH modulation antagonizes SA accumulation and SA-dependent responses. GSH acts on allowing increased intracellular ROS to activate SA signalling (Han et al., 2013). In this study, application of GSH or OTC or overexpression of NbECS markedly increased SA levels and the expression of NbICS1 (SA biosynthetic gene), NbNPR1 (SA signalling gene), and SA-mediated defence genes such as NbPR1, NbPR2, and NbPR5. However, silencing of NbECS or NbGS or treatment with BSO led to markedly decreased SA levels, and decreases in the expression of Nbics1, NbNPR1, NbPR1, NbPR2, and NbPR5. Therefore, these results indicate that GSH has a primary role in resistance to TMV, and GSH is essential for both local and systemic resistance against TMV infection through modulation of SA signalling.

An early ROS production during TMV infection could contribute to resistance. Several studies have shown that an early (from 6–10 h after inoculation) accumulation of the ROS has a role in hypersensitive response (HR)-associated (localized necrosis) resistance to plant viruses like TMV (Doke & Ohashi, 1988; Rossetti & Bonatti, 2001). Various biotic and abiotic stresses may result in the over-accumulation of ROS, which can cause damage to plants (Mittler, 2002). The production of ROS is often correlated with plant cell death and enhanced susceptibility to pathogens (Mittler et al., 2004). Our results showed that silencing of NbECS or NbGS or treatment with BSO led to markedly decreased ROS accumulation and, accordingly, enhanced susceptibility to TMV infection. However, application of GSH, OTC, or overexpression of NbECS resulted not only in increased resistance to TMV but also in a decrease of ROS accumulation in later phases of viral pathogenesis (3 dpi). ROS are continuously produced in plants and cause potential toxic effects to DNA, RNA, proteins, and membrane oxidation in plants. In fact, low levels of ROS could enhance tolerance during plant defence responses against various types of stresses (Baxter et al., 2014; Hafez

**FIGURE 8** A working model for the role of glutathione (GSH) in the plant defence signalling network against viral infection. Viral infection results in an increase in the expression of γ-glutamylcysteine synthetase (ECS) and GSH synthetase (GS), which promotes GSH biosynthesis, is required for the activation of ISOCHORISMate SYNTHASE 1 (ICS1)-dependent salicylic acid (SA) accumulation, downstream NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) signalling processes, and reactive oxygen species (ROS) scavenging-related genes. As the expression of ROS scavenging-related genes are elevated, the down-regulation of ROS level alleviates viral symptoms. An early ROS burst, followed by suppression of ROS, is a requirement for successful resistance of plants to tobacco mosaic virus. Meanwhile, nuclear translocation of NPR1 together with an increased SA concentration promotes activation of the transcription of defence genes and increases resistance to virus invasion. TGAs, transcription factors of the TGA family

with OTC in *N. tabacum* decreases the number of necrotic lesions and TMV content (Gullner et al., 1999). However, when OTC was applied to PPV-infected peach plantlets, the OTC treatments did not reduce the virus content, although GSH levels were increased (Clemente-Moreno et al., 2012). Therefore, the mechanisms of this antiviral action are complex and varied. In this study, application of GSH or OTC on the primary (local) leaves reduced TMV accumulation in the inoculated leaves and noninoculated upper leaves. On the other hand, GSH synthesis can be suppressed by BSO, which selectively inhibits the activity of γ-glutamylcysteine synthetase (Senda & Ogawa, 2004; Zechmann et al., 2006). However, studies of the role of GSH in the plant defence network against viral infection in the presence of BSO have rarely been reported. Our results indicated that treatment with BSO on the primary (local) leaves of *N. benthamiana* caused more TMV accumulation in inoculated leaves and systemic upper leaves. Application of GSH or OTC increased systemic resistance, whereas treatment with BSO compromised systemic resistance to TMV infection. Transgenic *N. tabacum* plants with high GSH content display an improved defence response to bacterial and
et al., 2012; Xu et al., 2014). Increasing evidence suggests that ROS also function as central signalling molecules during plant defence responses and play an important role in the plant disease resistance (Dat et al., 2000; Ge et al., 2015; Levine et al., 1994; Pogány et al., 2009; Torres, 2010; Torres et al., 2005). Plants have evolved complicated scavenging and regulation systems to monitor ROS (Dat & Roychoudhury, 2014). The potential for cellular damage from ROS has been alleviated through evolutionary pressure to develop and expand a range of enzymatic and nonenzymatic ROS scavengers (Waszczyk et al., 2018). ROS-scavenging enzymes, such as GST, CAT, SOD, GPX, and enzymes related to the ascorbate–glutathione cycle, play significant roles in maintaining normal cellular ROS homeostasis. The present data revealed that GSH is a positive regulator of virus resistance and ROS scavenging processes in response to TMV infection in N. benthamiana plants. In fact, treatment with BSO markedly suppressed the expression of many ROS-scavenging genes. However, application of GSH, OTC or overexpression of NbECS led to the up-regulation of many ROS-scavenging genes. Taken together, artificial elevation of the GSH level can increase viral resistance by increasing the cell membrane stability and maintaining redox homeostasis. Therefore, it is proposed that GSH plays a positive role in N. benthamiana resistance to TMV infection by controlling the expression of downstream genes involved in the ROS-scavenging pathway.

In conclusion, our results demonstrate a critical role for GSH in the activation of SA production, downstream signalling pathways, and modulation of ROS homeostasis through controlling the expression of ROS-associated genes in plant defence responses (Figure 8). We provide evidence that the elevation of GSH leads to up-regulating SA biosynthesis and signalling pathways, PR defence-related genes, and ROS scavenging-associated genes, whereas the inhibition of GSH biosynthesis results in the down-regulation of the SA pathway, pathogenesis-related (PR) genes, and ROS-associated genes. Our model indicates that viral infection results in an increase in the expression of γ-glutamylcysteine synthetase and GSH synthetase, which promote GSH biosynthesis, and are necessary for SA accumulation, downstream signalling processes, and ROS scavenging-related genes activation (Figure 8). Increasing evidence indicates that an early ROS burst, followed by suppression of ROS, is a requirement for successful resistance of plants to TMV and other viruses (Balasubramaniam et al., 2014; Király et al., 2008; Shang et al., 2019; Zhu et al., 2020). Overall, in N. benthamiana GSH is essential for both local and systemic resistance against TMV infection through a differential modulation of SA signalling and ROS, possibly acting as the initiating signals for systemic resistance.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials and growth conditions

The WT N. benthamiana and NbECS-transgenic N. benthamiana plants and were grown in a greenhouse at 25°C and 16 h light/8 h dark cycles (100 μmol-m⁻²-s⁻¹). Six- to 7-week-old seedlings were used in the experiments.

4.2 | Virus inoculation, GFP imaging, and chemical treatments

Reduced GSH was purchased from Sangon Biotech, OTC was purchased from Tokyo Chemical Industry, and BSO was purchased from Aladdin. GSH, OTC, and BSO solutions were prepared in distilled water. The chemicals and the concentrations used were GSH 1 mM, GSH 10 mM, OTC 1 mM, OTC 10 mM, BSO 0.1 mM, and BSO 1 mM. Distilled water was used as a control treatment. TMV-GFP was maintained in an aqueous suspension of 0.02 M phosphate-buffered saline (PBS) at 4°C. The inoculation with TMV-GFP was performed using the method reported previously (Zhu et al., 2016). N. benthamiana plants were treated at one site on the primary (local) leaves with distilled water, GSH, OTC, or BSO for 2 days, then inoculated with TMV-GFP on the secondary (systemic) leaves. GFP fluorescence was photographed under ultraviolet (UV) light using a B-100AP longwave-UV lamp (Ultra-Violet Products) (Zhu et al., 2014, 2016). The GFP fluorescent foci quantification analysis (the ratio of GFP fluorescent area to the total area) was determined using ImageJ software (http://rsb.info.nih.gov/ij/).

4.3 | Cloning of complete NbECS and NbGS genes

The complete ORF of NbECS (γ-glutamylcysteine synthetase) and NbGS (glutathione synthetase) was cloned from N. benthamiana plants by PCR using the specific primers based on the template SIGSH1 (GenBank accession no. AF017983) and SIGSH2 (GenBank accession no. AF017984), respectively. These primers are listed in Table S1. The sequences of NbECS and NbGS were submitted to GenBank (GenBank accession nos. MW014353 and MW014352).

4.4 | Measurement of GSH content

GSH content assay was measured by using a commercially available kit (Solarbio) and all assays were performed in strict accordance to the kit protocol. A completely randomized design with three biological replicates was performed in each experiment.

4.5 | H₂O₂ and O₂•− visualization and determination

The accumulation level of H₂O₂ was observed using DAB as described previously (Zhu et al., 2020). Endogenous H₂O₂ concentrations were determined according to the protocol described by Chen et al. (2019) using a hydrogen peroxide assay kit (Solarbio) (Chen et al., 2019). Qualitative test of O₂•− was observed using NBT with a similar procedure to that described previously (Zhu et al., 2020). The content of O₂•− in N. benthamiana leaves was measured according to
previously methods as described by Cai et al. (2018) using a Micro Superoxide Anion Assay Kit (Solarbio) (Cai et al., 2018).

4.6 Electrolyte leakage and MDA content determination

Electrolyte leakage and MDA content measurements were performed as described previously (Zhu et al., 2020).

4.7 Construction of VIGS vectors

To construct the VIGS vector, partial cDNA of NbECS and NbGS was amplified by RT-PCR from a cDNA library of N. benthamiana leaf tissues using the gene-specific primers. The primers are shown in Table S1. Then, RT-PCR products were cloned into pCR8/GW/TOPO from a TOPO TA cloning kit (Invitrogen), according to the instructions of the manufacturer. Then, the partial fragments of the NbECS and NbGS genes were inserted into TRV vector pTRV-RNA2.

4.8 TRV-mediated VIGS assay

For the VIGS assay, pTRV1, pTRV2, pTRV-NbECS, and pTRV-NbGS were introduced into Agrobacterium tumefaciens GV2260 by electroporation. Further details are as described previously (Zhu et al., 2014).

4.9 RNA extraction, RT-PCR, and RT-qPCR

Total RNAs were isolated from N. benthamiana leaves as previously described (Zhu et al., 2020). For RT-PCR, the first-strand cDNA was prepared using M-MLV reverse transcriptase (Takara). To test the effectiveness of VIGS in the gene-silenced plants of NbECS and NbGS, RT-qPCR (Wangdi et al., 2010; Zhu et al., 2019) was used to detect the expression levels of NbECS and NbGS using the unique 5' region of NbECS and NbGS with specific primers. Primers that anneal outside the region targeted for silencing were used to ensure that only the endogenous gene could be tested. To compare gene expression differences, RT-qPCR analyses were performed to detect the expression levels of NbECS, NbGS, NbIC51, NbNPR1, NbPR1, NbPR2, NbPR5, NbAPX1, NbAPX2, NbAPX3, NbAPX4, NbAPX5, NbAPX6, NbAPX7, NbCAT1, NbCAT2, NbCAT3, NbCu/Zn-SOD, NbFeSOD, NbMnSOD, and TMV-MP. RT-qPCR analysis was performed on an iCycler (Bio-Rad). Relative quantification of the target gene expression level was performed using the comparative C_t (threshold cycle) method (Zhu et al., 2014). Three technical replicates were performed for each experiment. The sequences of RT-PCR and RT-qPCR primers are listed in Table S1. Amplification of the actin gene was used as an internal control according to a previous study (Zhu et al., 2020).

4.10 Protein extraction and western blot analysis

Total proteins were extracted with extraction buffer (50 mM Tris, Cl, pH 6.8, 5% mercaptoethanol, 10% glycerol, 4% SDS, 4 M urea) in an ice bath. Western blot analysis was performed according to a previous study (Zhu et al., 2013). The origin of the TMV-CP antibody used in these experiments was from Professor Wong Sek Man (Department of Biological Sciences, National University of Singapore).

4.11 SA determination

The SA content was quantified by high-performance liquid chromatography–mass spectrometry (HPLC-MS) from crude plant extracts according to the method of Pan et al. (2010). The d_4-SA (2-hydroxybenzoic acid-[^4]H_4) was obtained from Sigma-Aldrich as internal standard.

4.12 Plasmid construction and plant transformation

The ORF of NbECS was amplified by RT-PCR using the specific primers NbECS-1300-F (5’-ACACGGGGGACGAGCTCGGTACCATGGCCTTGATGTCT-3’) and NbECS-1300-R (5’-GCCCTTGCTACCTACGTTCGACGTAGAGAAGCTCCTCAAAGA-3’), which incorporate restriction sites KpnI or SalI between gene and vector sequence. The amplified product was cloned into the pCAMBIA1300 plant binary vector driven by the cauliflower mosaic virus (CaMV) 35S promoter. Homologous recombination is the principle of the connection between gene and vector. The connection step was done through the In-Fusion HD Cloning Kit (Takara). The plasmid pCAMBIA1300-NbECS carries the kanamycin resistance (Kan) selectable marker in plants. The plasmid pCAMBIA1300-NbECS was transformed into A. tumefaciens EHA105 by electroporation. NbECS-transgenic N. benthamiana plants were generated by leaf disc transformation through an Agrobacterium-mediated method, as previously described (Zhu et al., 2020).

4.13 Molecular analysis of NbECS-transgenic N. benthamiana lines

Total RNA was isolated from positive lines as well as from the non-transformed WT N. benthamiana plants. Overexpression of NbECS in the transgenic plants was confirmed by semiquantitative RT-PCR and RT-qPCR. Semiquantitative RT-PCR was first performed to detect the expression levels of the hygromycin gene using 1 µg of total RNA isolated from WT N. benthamiana and NbECS-transgenic N. benthamiana plants. RT-qPCR was used to detect the expression levels of the NbECS using 1 µg of total RNA isolated from WT
**N. benthamiana** and NbECS-transgenic **N. benthamiana** plants. Actin was used as the internal reference gene.

### 4.14 Statistical analysis

The values are presented as mean ± SD of at least three replicates. Significance of differences were analysed by two-tailed Student’s t test between two groups and by one-way analysis of variance followed by Tukey test between multiple groups. Asterisks indicate the different statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Supporting Information**

Additional Supporting Information may be found in the online version of the article at the publisher's website.

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