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ORIGINAL ARTICLE

Nicotiana tabacum overexpressing γ-ECS exhibits biotic stress tolerance likely through NPR1-dependent salicylic acid-mediated pathway

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Abstract The elaborate networks and the crosstalk of established signaling molecules like salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), reactive oxygen species (ROS) and glutathione (GSH) play key role in plant defense response. To obtain further insight into the mechanism through which GSH is involved in this crosstalk to mitigate biotic stress, transgenic *Nicotiana tabacum* overexpressing *Lycopersicon esculentum* gamma-glutamylcysteine synthetase (*LeECS*) gene (*NtGB* lines) were generated with enhanced level of GSH in comparison with wild-type plants exhibiting resistance to pathogenesis as well. The expression levels of non-expressor of pathogenesis-related genes 1 (NPR1)-dependent genes like pathogenesis-related gene 1 (*NtPR1*), mitogen-activated protein kinase kinase (*NtMAPKK*), glutamine synthetase (*NtGLS*) were significantly enhanced alongwith *NtNPR1*. However, the expression levels of NPR1-independent genes like *NtPR2*, *NtPR5* and short-chain dehydrogenase/reductase family protein (*NtSDRLP*) were either insignificant or were downregulated. Additionally, increase in expression of thioredoxin (*NtTRXh*), *S*-nitrosoglutathione reductase 1 (*NtGSNOR1*) and suppression of isochorismate synthase 1 (*NtICS1*) was noted. Comprehensive analysis of GSH-fed tobacco BY2 cell line in a time-dependent manner reciprocated the *in planta* results. Better tolerance of *NtGB* lines against biotrophic *Pseudomonas syringae* pv. *tabaci* was noted as compared to necrotrophic *Alternaria alternata*. Through two-dimensional gel electrophoresis (2-DE) and image analysis, 48 differentially expressed spots were identified and through identification as well as functional categorization, ten proteins were found to be SA-related. Collectively, our results suggest GSH to be a member in cross-communication with other signaling molecules in mitigating biotic stress likely through NPR1-dependent SA-mediated pathway.

Keywords Biotic stress · Glutathione · *Nicotiana tabacum* · NPR1 · Salicylic acid · Transgenic plants

Abbreviations

ET	Ethylene
GSH	Glutathione
JA	Jasmonic acid
NPR1	Non-expressor of pathogenesis-related genes 1
PR	Pathogenesis-related protein gene
ROS	Reactive oxygen species
SA	Salicylic acid

Introduction

A broad range of functions of GSH in plants, including redox control, detoxification of heavy metals and electrophilic xenobiotics, serving as electron donor for biochemical reactions, long-distance transport of reduced sulfur, stress defense gene expression, direct posttranslational modifications of proteins through reversible glutathionylation of thiol residues etc. have been reported so far (Foyer

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et al. 1997; May et al. 1998; Ball et al. 2004; Pasternak et al. 2008; Ishikawa et al. 2010; Mhamdi et al. 2010). In addition, GSH in relation to tolerance of abiotic stresses like frost, salt, chilling, heavy metal and so on has been studied as well (Kocsy et al. 2000; Ruiz and Blumwald 2002; Gomez et al. 2004; Kumar et al. 2009). Appertaining to the aforementioned functions, the potential role of GSH has been investigated in various defense reactions, demonstrating that GSH participates in plant defense reactions against viral, fungal and bacterial infections. Exogenous application of GSH was found to activate several genes encoding enzymes that participate in the biosynthesis of lignin and phytoalexins in bean cell culture (Wingate et al. 1988). It has long been known that, elicitor stimulation induces chalcone synthase expression, and this effect can be partly mimicked by GSH supplementation (Dron et al. 1988). Treatment of bean and soybean cells with fungal elicitor or GSH causes the rapid insolubilization of hydroxy-proline-rich structural proteins in the cell wall (Bradley et al. 1992). Early study revealed that, the enhanced resistance of melon and tomato roots against *Fusarium oxysporum* brought about by herbicides coincides with the significant increase of GSH levels (Bolter et al. 1993). In compatible barley–barley powdery mildew interactions the ascorbate-GSH cycle and other antioxidative enzymes (e.g. glutathione *S*-transferase) are activated and these processes might diminish the damaging effects of oxidative stress. However, in incompatible interactions these antioxidative reactions are not or are only slightly activated (El-Zahaby et al. 1995). A considerable accumulation of GSH and, in particular, oxidized glutathione (GSSG) has been observed in tomato cells carrying *Cf-9* or *Cf-2* resistance genes after treatment with race-specific elicitors of the fungus *Cladosporium fulvum* (May et al. 1996). Additionally, previous studies also reported that *Arabidopsis pad2-1* mutant with only 22% of wild-type amounts of GSH were susceptible to *P. syringae* as well as *Phytophthora brassicae* (Glazebrook and Ausubel 1994; Glazebrook et al. 1997; Parisy et al. 2007). These results cumulatively suggest that the accumulation of GSH may be necessary for disease resistance.

Genetic, physiological and molecular analyses have revealed that the stress-related phytohormones SA, JA and ET play key roles in the complex crosstalk among signaling pathways to mitigate biotic stress (Thomma et al. 1998; Glazebrook 2001; Grant and Jones 2009; Pieterse et al. 2009). The small plant hormone molecule SA plays significant role in plant disease resistance. Active oxygen species has also been found to be involved in SA-mediated systemic acquired resistance (Chen et al. 1993). Considering the interaction of SA with other established signaling molecules, antagonism between SA and JA is a widely accepted fact, synergistic actions of SA and JA/ET have been reported as well (Van Wees et al. 2000; Mur et al. 2006), highlighting the potential significance of SA-JA crosstalk in nature (Leon-Reyes et al. 2010). Further, SA signaling is classically thought to control the resistance to biotrophic pathogens; by contrast, JA and ET-signaling pathways are responsible for resistance to necrotrophic pathogens (Glazebrook 2005; Loake and Grant 2007).

Studies have shown that innate immune responses are associated with changes in cellular redox states sensed by NPR1 (Mou et al. 2003; Tada et al. 2008). After detecting microbial pathogens, plants accumulate SA, subsequently activates NPR1, which play an important regulatory role in plant defense including the expression of *PR* genes (Cao et al. 1994, 1997; Zhang et al. 1999; Zhou et al. 2000; Pieterse and Van Loon 2004). Increase in SA triggers the reduction of disulphide bonds located on NPR1. This reduction stimulates both the translocation of NPR1 from the cytosol to the nucleus and the physical interaction of NPR1–TGA1 that is necessary for the activation of *PR* gene transcription (Mou et al. 2003; Després et al. 2003). Previous study also showed that NPR1 plays a crucial role in the cross-communication between SA-JA-dependent defense signaling pathways (Spoel et al. 2003). In addition, the SA-induced NPR1 oligomer-to-monomer reaction regulated by thioredoxin through reduction of its intermolecular disulphide bonds has also been reported (Tada et al. 2008). Further, SA signaling is thought to be mediated by at least two mechanisms, one requiring NPR1 and a second that is independent of NPR1 (Blanco et al. 2009).

In contrast to the overwhelming amount of information with respect to SA, JA, ET, ABA and ROS as well as their crosstalk serving as important regulators of induced disease resistance, the role of GSH cross-communication networks in plant defense is less well understood. The aim of the present study was to obtain an insight into the role of GSH in the crosstalk with other signaling molecules in mitigating biotic stress. Here, the genetic engineering approach has been used to develop and establish transgenic tobacco overexpressing *LeECS* with enhanced GSH content. Biotic stress tolerance potential of the *NtGB* lines has also been established. Transcript profiling of selected defense-related genes of the transgenic as well as the wild-type plants and a time-course gene expression analysis of GSH-fed BY2 cell line have been determined. Until now, limited data are available about the stress-elicited changes in plants with enhanced GSH content, at the proteome level. Proteome analysis of the *NtGB* lines and identification of changes at the protein expression level have been performed. Taken together, present findings suggest that GSH plays an important role in biotic stress tolerance with a possible crosstalk through NPR1-dependent SA-mediated signaling pathway.

Materials and methods

Plant materials and growth conditions

Tobacco (*N. tabacum* cv. Xanthi) and *L. esculentum* seeds were sown on MS medium (Murashige and Skoog 1962) containing 3% sucrose and 0.8% agar and allowed to germinate at 22 ± 1°C (16-h of 150 μE m^{-2} s^{-1} light and 8-h darkness). Subsequently plants were grown and maintained in MS medium supplemented with 1 mg L^{-1} IBA and 0.25 mg L^{-1} kinetin. Tobacco BY2 cells were grown in dark on modified MS medium to which KH_2PO_4, thiamine and myo-inositiol were added in excess. The medium was supplemented with 0.2 mg L^{-1} 2, 4-D.

Plasmid construction and plant transformation

A cDNA designated as *LeECS* was isolated from 2-week-old *L. esculentum* seedlings by RT-PCR. Total RNA was isolated using Trizol reagent (Invitrogen, USA) and first-strand cDNA was synthesized from total RNA using RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, USA) with oligo$(dT)_{18}$ primer as standardized by Banerjee and Chattopadhyay (2010). The 1,571-bp full-length DNA sequence of *LeECS* was amplified using gene-specific primers: forward, 5′-ATCTAGATATGGCCTTGATGTCT CAGGC-3′; and reverse, 5′-AAGAGCTCAATCAGTAGA GAAGCTCCTCAAA-3′ (Sigma, USA). The PCR product was cloned into pGEM-T Easy vector (Promega, USA) and subcloned into the binary vector pBI121 at *Xba*I and *Sac*I sites. Tobacco leaf discs were infected with *Agrobacterium tumefaciens* LBA4404 harboring the recombinant plasmid. The regenerated shoots were maintained on MS medium supplemented with 0.1 mg L^{-1} NAA and 1 mg L^{-1} BAP alongwith 50 mg L^{-1} kanamycin and 62.5 mg L^{-1} cephotaxime. Kanamycin-resistant and PCR positive, transgenic T_0 plants were selected, and transferred to greenhouse and maintained up to T_2 generation, which were used for further analysis.

Molecular analysis of *NtGB* lines

PCR was employed to screen the transformants carrying *npt*II gene. Genomic DNA was extracted from young leaves of wild-type and *NtGB* lines by cetyltrimethylammonium bromide (CTAB) method. PCR amplification was performed as follows: 94°C for 45 s, 55°C for 45 s and 72°C for 1 min, with an initial denaturation at 94°C for 1 min using the primer pair listed in Supplementary Table S1.

For RT-PCR, total RNA from tobacco leaves was isolated and cDNA synthesized as mentioned earlier. *Npt*II and *LeECS* transcripts were detected by semiquantitative RT-PCR using specific primers (Supplementary Table S1). PCR amplification of *npt*II was performed as mentioned earlier and amplification of *LeECS* was performed as follows: 94°C for 45 s, 63°C for 30 s and 72°C for 1 min, with an initial denaturation at 94°C for 5 min. Equal loading for amplification of each cDNA was determined by *actin* PCR product.

For Southern blot analysis, a total of 10 μg of genomic DNA was digested with *Eco*RI and *Hind*III separately, fractionated on 0.8% agarose gel and then transferred onto Immobilon-NY^+ membrane (Millipore, USA). Hybridization was performed at 68°C using a 700-bp fragment of *LeECS* as probe, labeled with [α-^{32}P] dATP. The blots were washed repeatedly under stringent conditions and exposed to X-ray film. All molecular biology experiments were performed following standard protocols (Sambrook and Russell 2001).

Determination of GSH and GSH:GSSG ratio

GSH was extracted from mature tobacco leaves and quantified (Tsakraklides et al. 2002). High-performance liquid chromatography (HPLC) was conducted using a 515-HPLC pump (Waters, USA) and 2475 fluorescence detector (Waters, USA), at a flow-rate of 1.5 mL min^{-1} using AccQ.Tag (3.9 × 150 mm) column (Waters, USA) at excitation wavelength of 360 nm and emission wavelength of 450 nm. Briefly, the elution condition was: solvent A, composed of sodium acetate and triethylamine at pH 5.05 at 5% (v/v) dilution and acetonitrile:water (30:70) as solvent B. From 0 to 9 min, A was 94%, from 9 to 16 min a linear gradient of 94 to 91.5% A was applied, from 16 to 22 min a linear gradient of 75% A was applied and from 22 to 30 min A decreased to 0% linearly. GSH (Sigma, USA) was used as standard. Data analyses were performed with Empower 2 software.

GSH:GSSG ratio was measured according to Ishikawa et al. (2010) with *NtGB* lines and wild-type plants. Total glutathione was measured by glutathione reductase (GR) recycling system coupled with 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) by measuring 5′-thio-2-nitrobenzoic acid (TNB) formation at 412 nm. GSSG was measured by pretreating the samples with 2-vinylpyridine to mask GSH and assayed in the presence of GR and DTNB as above. GSH was calculated by subtracting the GSSG content from the total glutathione content and GSH:GSSG ratio was determined as well.

Extraction and quantification of SA

Extraction and quantification of SA was performed as described previously (Freeman et al. 2005). SA was quantified by HPLC with a fluorescence detector, as mentioned

earlier using Symmetry C-18 reverse-phase column (5 μm, 4.6 × 250 mm) at excitation wavelength of 254 nm and emission wavelength of 395 nm. The elution condition was methanol gradient (solvent A, water and 1% formate; and solvent B, 100% methanol and 1% formate) of 10 to 40% B (10 min), 40 to 50% B (5 min), 50 to 100% B (2.5 min), 100 to 40% B (2.5 min), 40 to 10% B (1 min) and 10% B (1 min) with a flow-rate of 1 mL min^{-1} over 22 min.

Treatment of tobacco BY2 cells

Cell suspension culture was established and treated with 100 μM GSH. GSH-induced as well as control cells were harvested after 3, 6 and 12 h following treatment, centrifuged briefly to collect cells for total RNA isolation. Total RNA was isolated using Trizol reagent as mentioned earlier.

Semiquantitative and quantitative real-time RT-PCR analysis

Semiquantitative RT-PCR was carried out using 1 μg total RNA isolated from *NtGB* lines and wild-type plants and cDNA synthesized as described previously. PCR amplification was performed for different genes at different cycles of 94°C for 30 s, 30 s at varying annealing temperatures for different genes, 72°C for 1 min, with an initial denaturation at 94°C for 1 min. Primer pairs used for semiquantitative RT-PCR are listed in Supplementary Table S1. *Actin* was used as a loading control. The PCR products were analyzed by 1% agarose gel electrophoresis.

Quantitative RT-PCR was performed using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, USA) with SYBR Premix Ex Taq (TaKaRa). Primer pairs are listed in Supplementary Table S1. Each PCR reaction contained 1× SYBR Premix *Ex Taq*, 0.05 nM of each primer, and 1 μL cDNA in a final volume of 20 μL. PCR amplification was performed for 40 cycles at 94°C, 30 s and 60°C, 2:30 min with a preceding initial denaturation of 30 s at 95°C. Relative expression levels were calculated by Δ-ΔCt method, and all quantifications were normalized using *actin* mRNA as an internal control.

Disease resistance assay of transgenic plants

Pseudomonas syringae pv. *tabaci* was grown up to an OD of 0.7, centrifuged and the cells were resuspended in 25 mL of 10 mM $MgCl_2$ (Pageau et al. 2006). The cells were injected subepidermally in two leaves of similar position from each of five independent wild-type and *NtGB* lines. The infection was monitored up to 14 days post inoculation (dpi). Leaf discs were ground in 10 mM $MgCl_2$, subjected to serial dilution and plated on 10 g L^{-1} yeast extract, 10 g L^{-1} peptone and 5 g L^{-1} NaCl containing 15 g L^{-1} agar (YEP) plates with rifampicin selection. For gene expression analysis, *P. syringae* pv. *tabaci* cells at particular ODs were used to infect wild-type and *NtGB* lines. Disease test with *A. alternata* (ITCC 6306) was performed according to Zhang et al. 2009a. Briefly, the fungus was grown for 7 days in petridishes containing potato dextrose agar (PDA) at 28°C in the dark. Agar plugs from actively growing culture of the fungus were excised and inverted on different positions of detached leaves and were placed on wet filter paper in petridishes and incubated at 28°C to permit normal disease development under high humidity. After 5 days, disease progression was checked, lesion diameter measured and photographed.

Western blotting

Proteins were extracted after homogenizing leaves in 50 mM potassium phosphate buffer, pH 7.8, containing 0.15% (v/v) Triton X-100 at 0°C. Protein samples were quantified by Bradford assay (Bradford 1976), using BSA as standard, resolved in 12% SDS-PAGE gels and transferred onto polyvinylidene difluoride membrane (Millipore, USA), blocked with 5% skimmed milk and gamma-glutamylcysteine synthetase (γ-ECS) protein bands were detected by using a rabbit polyclonal antibody raised against maize γ-ECS (Agrisera, Sweden) as the primary antibody and an anti-rabbit IgG conjugated to horseradish peroxidase (Sigma-Aldrich, USA) as the secondary antibody. Immunoreactive proteins were visualized using the SuperSignal West Pico (Pierce, USA) chemiluminescent reagent.

Protein extraction and 2-DE

Proteins from leaves of three biological replicates at similar positions of wild-type, *NtGB*9 and *NtGB*19 plants were extracted. Approximately 2.5 g of tissue samples was ground in liquid nitrogen and suspended in extraction buffer [700 mM Sucrose, 500 mM Tris–HCl, pH 7.5, 50 mM EDTA, 100 mM KCl, 2% (w/v) β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride] and protein extraction was done following the phenol extraction method (Isaacson et al. 2006). The resultant pellet was resuspended in 2-DE sample buffer consisting of 9 M Urea, 4% (w/v) CHAPS, 0.5% (v/v) Triton X-100, 20 mM dithiothreitol (DTT) and 1% (w/v) Bio-Lyte (3/10) ampholyte (BioRad Laboratories, Hercules, CA, USA) as standardized before (Sinha and Chattopadhyay 2010). The total protein concentration was analyzed by Bradford assay. 100 μg of total protein for each sample was used for passive rehydration of 7 cm IPG strip (pH 4–7; BioRad Laboratories, Hercules, CA, USA). Programmed IsoElectric Focusing (IEF) was

performed with the Protean IEF cell (BioRad Laboratories, Hercules, CA, USA) using the following conditions: 250 V for 15 min, 4,000 V for 7 h, 500 V for 5 min. The focused strips were equilibrated twice in Equilibration Buffer I and II, respectively (BioRad Laboratories, Hercules, CA, USA), for 15 min each. The equilibrated strips were then subjected to the second dimension, which was carried out in SDS-PAGE in a vertical slab of 12% Acrylamide in BioRad Mini-PROTEAN Tetra Cell. After running for 35 min at 200 V, gels were stained with colloidal Coomasie Brilliant Blue (CBB) G-250 (Neuhoff et al. 1988).

Image and data analysis

The gel images were acquired using Versa-Doc Image system (BioRad Laboratories, Hercules, CA, USA) and image analysis was performed with PD Quest software version 8.0.1 (BioRad Laboratories, Hercules, CA, USA). Detection of spots was performed by matching the gels automatically, followed by manual verification. Protein spots were annotated only if detected in all gels after normalization of the spot densities against the whole gel densities; the percentage volume of each spot was averaged for nine different (three biological replicates of wild-type and *NtGB* lines) gels and statistical analysis was performed to find out significant protein fold changes between wild-type and *NtGB* lines.

Protein identification using MALDI TOF-TOF MS/MS

Selected protein spots were excised from 2-DE gels and subjected to in-gel digestion with trypsin following the manufacturer's instructions (in-gel trypsin digestion kit, Pierce, USA). Digested proteins were further desalted with Zip-Tip μ-C18 (ZipTip, Millipore, USA) and analyzed using a 4800 MALDI TOF-TOF MS/MS analyzer (Applied Biosystems, Foster City, CA, USA). Peptides were evaporated with a ND:YAG laser at 355 nm, using a delayed extraction approach. They were accelerated with 25 kV injection pulse for TOF analysis. Each spectrum was the cumulative average of 1,000 laser shots. The MS/MS spectrum was collected in MS/MS 1 kV positive reflectron mode with fragments generated by post source decay (PSD). The MS/MS mass tolerance was set to $\pm$20 ppm. After processing, ten MS/MS precursor was selected (Minimum signal to noise ratio-50). Before each analysis, the instrument was calibrated with the Applied Biosystems 4700 Proteomics Analyzer Calibration Mixture. Data interpretation was carried out using the GPS Explorer Software (Applied Biosystems), and an automated database search was carried out using the MASCOT program (Matrix Science Ltd., London, UK).

MS/MS data was used to perform protein identification by searching in a non-redundant protein sequence database (NCBI nr—20070216; 4626804 sequences, 1596079197 residues) using a MOWSE algorithm as implemented in the MASCOT search engine version 3.5 (Matrix science: http://www.matrixscience.com). The following parameters were used for database searches: taxonomy, viridiplantae (green plants; 186963 sequences); cleavage specificity, trypsin with one missed cleavages allowed; mass tolerance of 100 ppm for precursor ions and a tolerance of 0.2 da for the fragment ions; allowed modifications, carbamidomethyl (fixed), oxidation of Met (variable), cleavage by trypsin, cuts C-term side of KR unless next residue is P. According to MASCOT probability analysis, only significant hits ($P < 0.05$) were considered.

To evaluate the functional categories and hierarchies of identified proteins, KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) was used (Kanehisa and Goto 2000). The theoretical peptide mass and pI of the polypeptides were evaluated at EXPASy (http://www.expasy.org/tools/pi_tool.html) (Bjellqvist et al. 1993; Gasteiger et al. 2005) for final confirmation according to their positions in the 2-DE gel map.

Statistical analysis

All experiments were repeated at least three times, unless otherwise stated, using plants grown independently. Significance of differences between data sets was evaluated by Student's t test (5% significance, $P < 0.05$). Calculations were carried out with Microsoft Excel software.

Results

Characteristics of *NtGB* lines

GSH is synthesized in two steps in which γ-ECS catalyzes the first step and is known to be the rate-limiting enzyme (Hell and Bergmann 1990; May et al. 1998). The gene encoding *L. esculentum* γ-*ECS* was cloned into the binary vector pBI121 under the control of CaMV35S promoter (Fig. 1a) and used to transform tobacco (cv. Xanthi) leaves. Kanamycin-resistant and PCR positive transgenic plants constitutively expressing 35S::*LeECS* were selected. Independent T_2 transgenic lines grown in greenhouse conditions were screened by PCR analysis (Fig. 1b). Semiquantitative RT-PCR analysis demonstrated that the levels of *npt*II and *LeECS* were increased in multiple T_2 lines (Fig. 1c). Southern blot analysis confirmed the stable integration of *LeECS* in T_2 lines (Supplementary Fig. S1a) and the LeECS protein was detected in significantly higher level in leaves of *NtGB* plants compared with that of

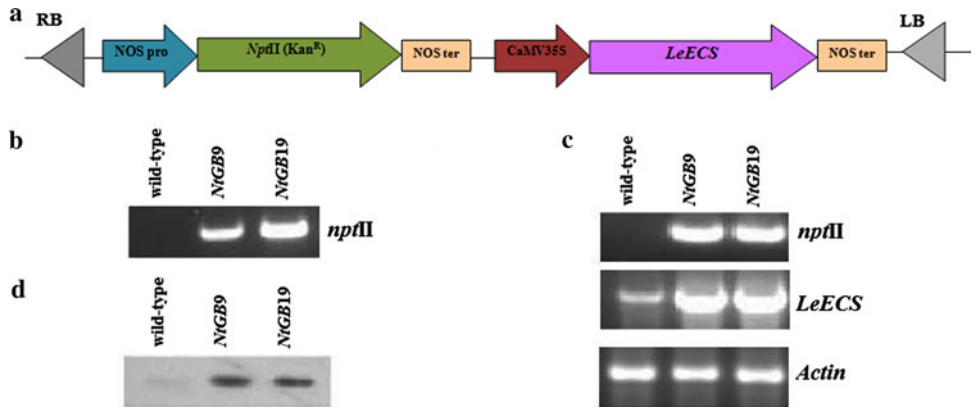


Fig. 1 Analysis of *NtGB* lines. **a** Construct design of 35S::*LeECS* that was inserted into the tobacco genome to constitutively express *LeECS* in *NtGB* lines. *LeECS* was cloned into the binary vector pBI121 at *Xba*I and *Sac*I sites after removing the *gus* gene, under CaMV35S promoter and *nos* terminator. **b** PCR screening of *npt*II in T_2 independently transformed *NtGB* plant lines grown in greenhouse conditions. Leaves from 6-week-old plants were used for analysis. **c** Semiquantitative RT-PCR analyses of wild-type and *NtGB* lines harboring *LeECS*. Expression of *npt*II and *LeECS* mRNA in different *NtGB* lines is shown. *Actin* was used as a loading control. **d** Western blot analysis showing the LeECS protein levels in wild-type and *NtGB* lines. γ-ECS protein bands were detected by western blotting using specific antibody

wild-type plants which showed lesser reactivity (Fig. 1d). The growth and appearance of *NtGB* were almost the same as those of wild-type plants throughout the growth period under normal growth conditions (data not shown). These findings suggest that enhanced GSH content through the overexpression of *LeECS* is not directly involved with the phenotypic alterations (Noctor et al. 1998; Zhu et al. 1999; Gullner et al. 2001; Xiang et al. 2001; Gomez et al. 2004; Liedschulte et al. 2010). Furthermore, previous study showed that transgenic plants having 500- to 1,000-fold increased SA and SAG accumulation did not show any phenotypic variation but constitutively expressed PR genes (Verberne et al. 2000) which can be corroborated by our study.

To determine the total glutathione content in *NtGB*9 and *NtGB*19 alongwith wild type, HPLC analysis was performed which revealed that the GSH content was 2.6- and 2.07-fold higher in *NtGB*9 and *NtGB*19, respectively (Supplementary Fig. S1b). These results clearly indicated that as a result of overexpression of *LeECS* in tobacco, GSH accumulation was enhanced in agreement with the findings reported previously (Noctor et al. 1998; Gomez et al. 2004). The GSH:GSSG ratio was 13.2 and 11.04 in *NtGB*9 and *NtGB*19, respectively, as compared with 8.8 in wild-type plant (Supplementary Fig. S1c). The leaves of *NtGB* lines were observed to accumulate more SA in comparison with that of wild-type (Supplementary Fig. S1d).

Transcript profiling in enhanced GSH condition

Gene expression profile of NtGB lines

To investigate the role of GSH in the signaling pathway crosstalk with SA, JA and ET in mitigating biotic stress, the expression profiles of selected genes involved with various defense-signaling pathways were taken into account. Experiments were also designed to understand the specific involvement of GSH with SA, and even if it is so, whether it follows NPR1-dependent or -independent pathway.

In this study, the expression level of *NtNPR1* was higher in *NtGB* lines as compared with the wild-type plants. The NPR1-dependent homologues in tobacco were also found to be enhanced as shown in Fig. 2. *PR1*, a marker for SA-mediated plant defense (Blanco et al. 2009); *GLS*, which catalyzes the first reaction in the main path of ammonia assimilation in higher plants and known to be induced upon infection with *P. syringae* (Pérez-García et al. 1995); *MAPKK*, a mitogen-activated protein kinase kinase that interacts with SA-induced protein kinase in tobacco, which may play a role in SA signaling through NPR1 (Liu et al. 2000); *TRXh*, which further catalyzes an SA-induced NPR1 activation by oligomer-to-monomer conversion in *Arabidopsis* (Tada et al. 2008); and *GSNOR1*, that catalyzes the catabolism of GSNO which further facilitates oligomerization of NPR1 in *Arabidopsis* (Tada et al. 2008) were considerably enhanced at transcript level. However, the transcript level of *NtICS1* was decreased in *NtGB* lines due to the suppression of its expression by nuclear NPR1 through a feedback loop, thus inhibiting the further elevation in the SA content in accordance with a recent report with *Arabidopsis* (Zhang et al. 2009b). Interestingly, the expressions of NPR1-independent genes like *NtPR2*, *NtPR5* and *NtSDRLP* were unchanged or downregulated (Fig. 3a). These findings suggest the role of GSH in mitigating biotic stress, which is likely to be regulated through NPR1-dependent SA-mediated pathway. However, further

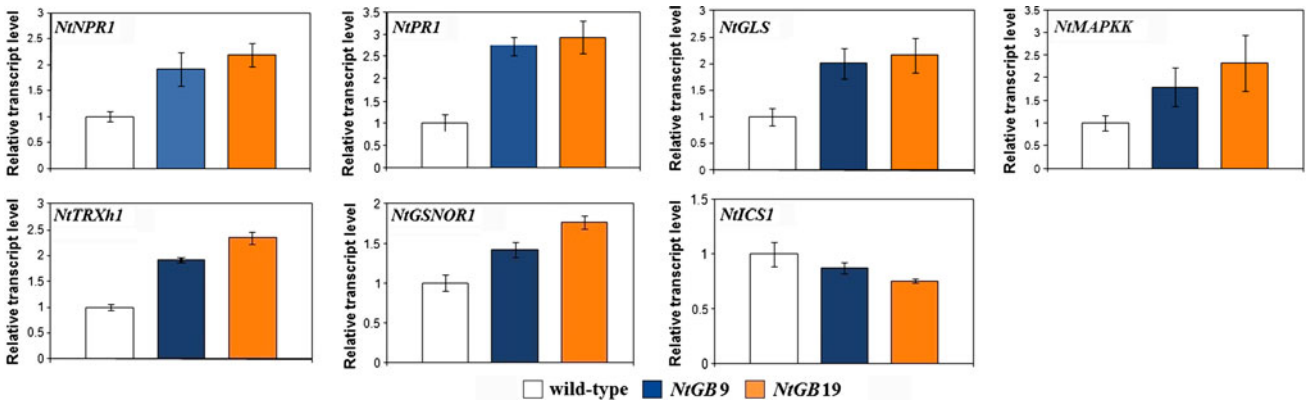


Fig. 2 Expression profile of NPR1-dependent SA-mediated pathway genes. Quantitative RT-PCR analysis was carried out to determine the expression levels of NPR1-dependent SA-mediated pathway. *Actin* was used as an internal control. Data are the mean ± SD for three individual experiments ($n = 3$) using plants grown independently

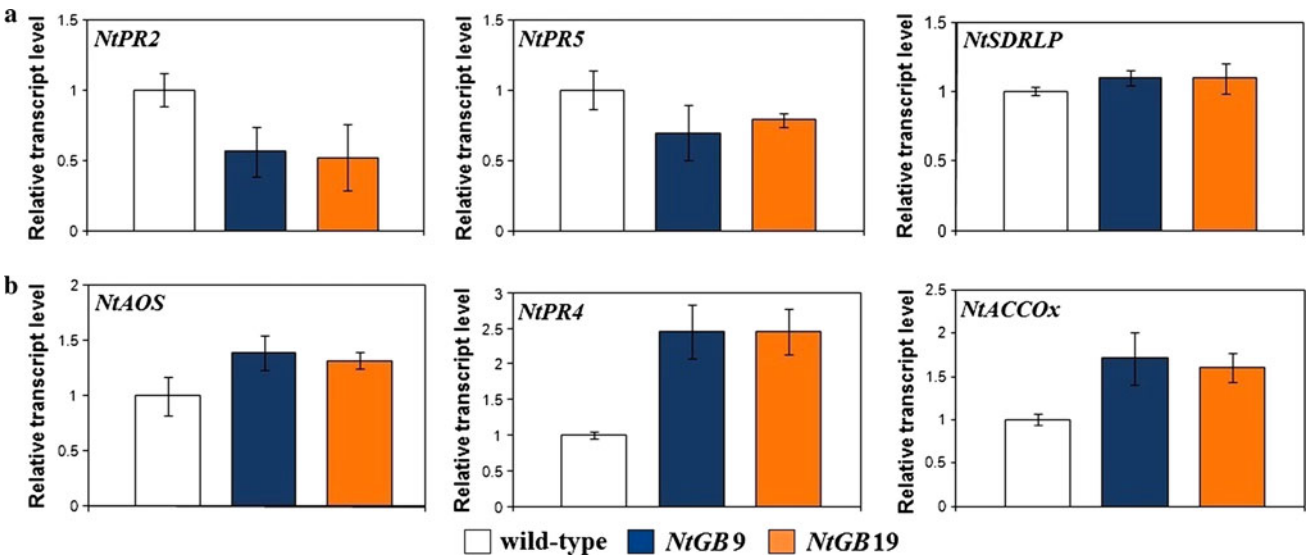


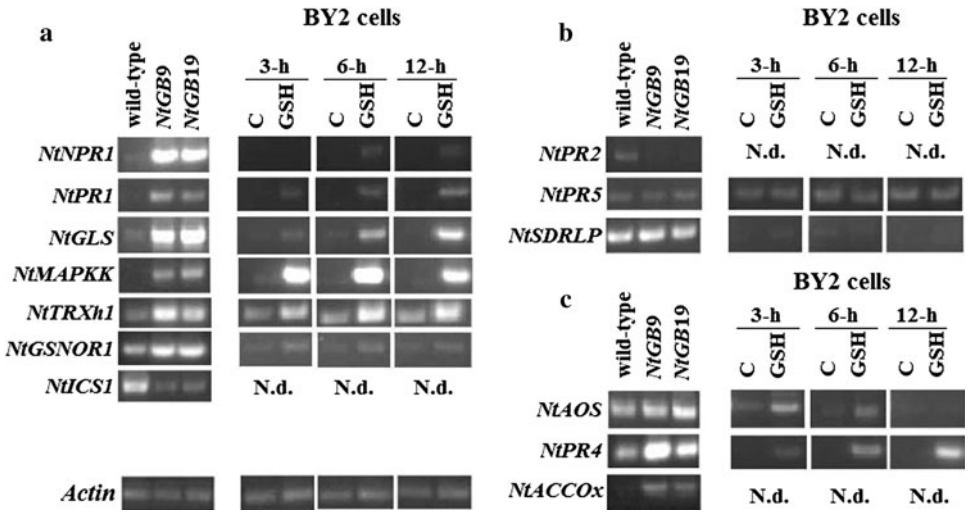
Fig. 3 Effect of *LeECS* overexpression on transcript levels of NPR1-independent SA-mediated pathway alongwith JA and ET pathway genes. **a** Quantitative RT-PCR analyses of NPR1-independent SA-mediated pathway genes. **b** Expression levels of *NtAOS* (enzyme of the first step in the biosynthesis of JA), *NtPR4* (ET marker) and *NtACCOx* (ET biosynthetic gene). *Actin* was used as an internal control. Data are the mean ± SD for three individual experiments ($n = 3$) using plants grown independently

pharmacological experimental evidences are required to confirm this.

To assess for any involvement of GSH with other signaling pathways viz. JA and ET, the expression pattern of allene oxide synthase (AOS) homolog in tobacco, an enzyme of JA biosynthetic pathway (Laudert and Weiler 1998) as well as *NtPR4*, the marker for ET signaling (Lawton et al. 1994) and ACC oxidase (ACCOx) that oxidizes ACC to ET was also monitored. The expression of *NtAOS* remained unchanged in *NtGB* lines, whereas, the expression level of *NtPR4* and *NtACCOx*, in *NtGB* lines was fairly increased as compared with the wild-type plants (Fig. 3b).

Gene expression profile of GSH-fed tobacco BY2 cells

To gain further understanding of the mechanisms of how GSH is responsible for the expression of the aforementioned set of genes, an experiment was designed on exogenously GSH-fed tobacco BY2 cells at transcript level in a time-dependent manner and compared with *in planta* results. To get exact comparative profile, semiquantitative RT-PCR analysis was performed which reciprocated the gene expression level with *NtGB* lines (Fig. 4). Interestingly, in GSH-fed tobacco BY2 cells the expression of *NtNPR1*, *NtPR1*, *NtGLS*, *NtMAPKK*, *NtTRXh*, *NtGSNOR1* and *NtPR4* were upregulated, whereas *NtPR5* and *NtSDRLP* were unchanged. *NtICS1*, *NtPR2* and



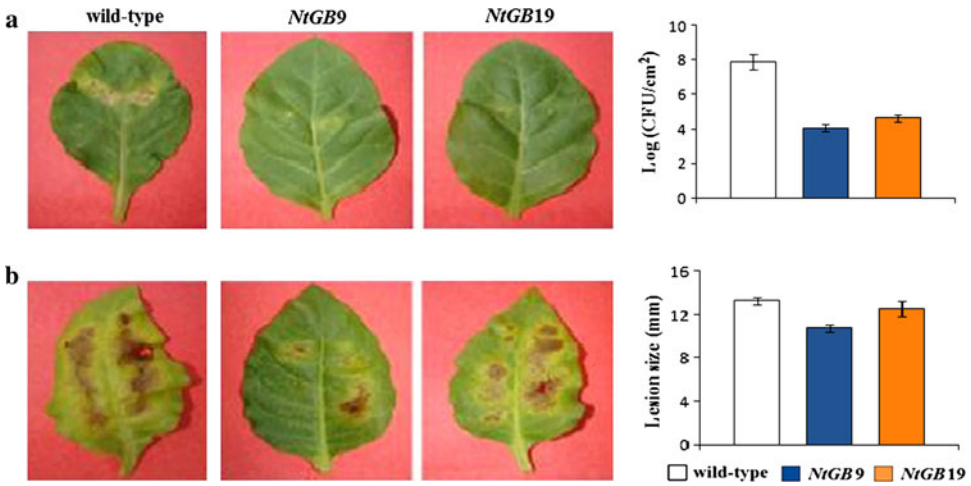


Fig. 4 Changes in the transcript levels of NPR1-dependent and -independent SA-mediated pathway, alongwith JA and ET pathway genes by the overexpression of *LeECS* and in GSH-fed tobacco BY2 cells. Semiquantitative RT-PCR analyses were carried out to determine the expression levels of **a** NPR1-dependent **b** NPR1-independent SA-mediated pathway. **c** Expression levels of *NtAOS*, *NtPR4* and *NtACCOx*. *Actin* was used as an internal control

Fig. 5 Disease resistance analysis of *NtGB* lines against the biotrophic and necrotrophic pathogens, *P. syringae* pv. *tabaci* and *A. alternata*. **a** Reduction of *P. syringae* pv. *tabaci* infection in whole plants of *NtGB* lines as compared with wild-type. Bacterial suspension was injected subepidermally and disease scored after 14 days. Bacteria were extracted from leaf discs, diluted and spread on YEP medium for colony count. **b** *A. alternata* actively growing culture was applied on different positions of detached leaves of *NtGB* and wild-type plants. Disease progression and lesion size was noted at 5 dpi. Data are the mean ± SD for five individual experiments ($n = 5$) using plants grown independently

NtACCOx were not detected at all. However, the expression level of *NtAOS* was decreased with time (Fig. 4).

Resistance patterns of *NtGB* lines to biotrophic *P. syringae* pv. *tabaci* and necrotrophic *A. alternata*

Whole plants of *NtGB*9 and *NtGB*19, exhibiting enhanced GSH contents, were infected with the biotrophic bacterial pathogen *P. syringae* pv. *tabaci*. On the other hand, necrotrophic fungus *A. alternata* was used to infect detached leaves of wild-type and *NtGB* lines. Enhanced resistance to *P. syringae* pv. *tabaci* was noted with *NtGB* lines in comparison with wild-type (Fig. 5a). In contrast, the disease progression of *A. alternata*-infected *NtGB*9 and *NtGB*19 was close to that of the wild type (Fig. 5b). These disease test assays support that SA-mediated defense response comes into play more prominently in *NtGB* lines.

A time-course study of gene expression was performed using *NtPR1, NtPR2, NtPR4* and *NtPR5* after infecting wild-type and *NtGB* lines with *P. syringae* pv. *tabaci*. Gene expression analysis of plants infected with *P. syringae* pv. *tabaci* grown up to an OD of 0.05 was monitored up to 48 h (Supplementary Fig. S2).

Proteomic analysis under enhanced GSH condition

Altered proteome profile in NtGB lines

To gain an insight into the nature of protein involved during GSH-mediated biotic stress tolerance, proteins

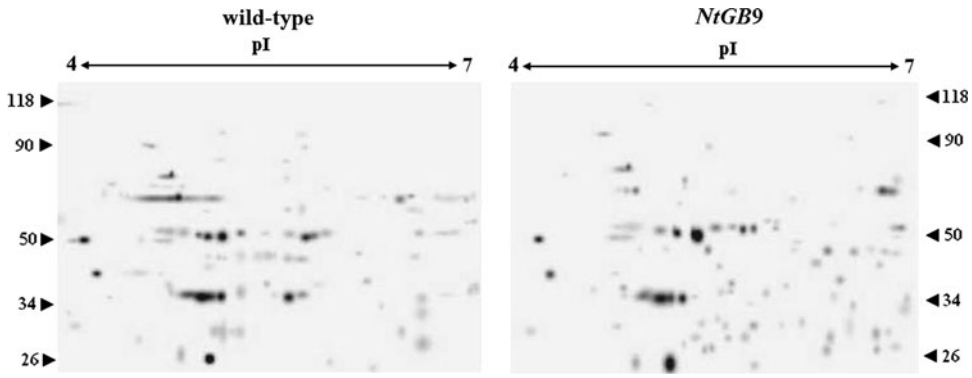


Fig. 6 Comparison of proteome patterns of *NtGB* lines and wild-type. Representative 2-DE gels of proteins extracted from young leaves of three biological replicates at similar positions of wild-type, *NtGB*9 and *NtGB*19 6-week-old plants

isolated from leaves of *NtGB*9, *NtGB*19 and that of wild-type plant were separated using 2-DE. Figure 6 represents gel images for both wild-type and *NtGB* samples in a pI range of 4–7. The results showed dramatic difference in global expression pattern of proteins in wild-type and *NtGB* lines. 48 spots were detected by statistical evaluation (Student's *t* test, at 5% significance, $P < 0.05$) of relative spot densities. These spots were further categorized as induced, i.e., uniquely expressed, upregulated (above twofold) and downregulated (below twofold).

Identification of differentially expressed proteins and functional classification

To identify the differentially expressed proteins, we used MALDI TOF-TOF MS/MS approach. The identified proteins for the corresponding 48 spots were sorted out into various categories. Protein functions were assigned according to KEGG and the functional categorization revealed that 38% of the differentially expressed proteins identified belong to the stress and defense category (Table 1; Figs. 7, 8). However, the classification of the protein is only tentative, since the biological function of many proteins identified has not yet been established experimentally.

The expression levels of six SA-related genes were confirmed by quantitative RT-PCR. For example, *NtPR10*, calmodulin-binding protein (*NtCBP*), arginine decarboxylase (*NtADC*) and carbonic anhydrase (*NtCA*) were found to be upregulated. These genes were therefore found to be possibly regulated at the transcription level as compared with others like heat-shock protein (*NtHsp70*), the expression of which was nearly unchanged and alcohol dehydrogenase (*NtADH*), which was downregulated (Fig. 9).

Discussion

Plant's resistance to pathogen attack is the consequence of a complex regulatory network that connects the individual signaling pathways, enabling each to assist or antagonize the others (Grant and Jones 2009; Pieterse et al. 2009). The role of GSH in plant defense has long been known (Dron et al. 1988; Wingate et al. 1988) in addition to substantial numbers of recent reports. A growing body of evidence also supports the notion that interplay between GSH and various established signaling components leads to the establishment of disease resistance. Current studies have also implicated that GSH is one of the important players in biotic stress management through its interaction with various established messengers. These prompted us to develop *NtGB* lines exhibiting enhanced GSH content, stable integration and higher expression of *LeECS* and more resistance to the biotroph *P. syringae* pv. *tabaci* in comparison with the necrotroph *A. alternata,* without any phenotypic variation to wild-type plants.

According to previous studies, higher level of GSH due to the overexpression of *γ-ECS* either of bacterial or of plant origin did not show any distinguishable difference in visual appearance in comparison with the wild-type plants as noted in poplar, mustard, *Arabidopsis* or tobacco (Noctor et al. 1998; Gullner et al. 2001; Zhu et al. 1999; Xiang et al. 2001; Gomez et al. 2004; Liedschulte et al. 2010), which can be corroborated by our results. However, some reports represented variation in phenotype as a result of overexpression of *γ-ECS* in tobacco and poplar, respectively (Creissen et al. 1999; Herschbach et al. 2000).

A recent report has shown that GSH status regulates SA and other pathways involved in biotic stress responses at several levels in *Arabidopsis*, including SA-accumulation and JA-linked gene expression (Mhamdi et al. 2010). Ball et al. (2004) reported that 32 stress-responsive genes were altered due to changed GSH metabolism in *Arabidopsis rax1-1* and *cad2-1*, mutants of γ-ECS. Additionally, previous studies also reported that *Arabidopsis pad2-1* mutant with only 22% of wild-type amounts of GSH were susceptible to *P. syringae* as well as *Phytophthora brassicae* (Glazebrook and Ausubel 1994; Glazebrook et al. 1997; Parisy et al. 2007). May et al. (1996) demonstrated a considerable accumulation of GSH and GSSG in tomato cells after treatment with race-specific elicitors of the

Table 1 Identification of proteins in *NtGB*9 D, Downregulated; U, upregulated; I, induced; SC, sequence coverage

Metabolic group	Spot no.	Theoretical mass (*kD*)/pI	Accession no.	SC %	Description	Fold change	Species
Stress and defense	U5701	25.3/9.25	15236687	29	VAMP7C (vesicle associated memb. Protein 7C)	2.84	*A. thaliana*
	U8101	31.3/6.01	27652184	17	Carbonic anhydrase 2	2.26	*F. bedentis*
	U5303	36.0/10.32	15487979	27	NBS/LRR resistance protein like protein	1.64	*T. cacao*
	U6701	2.89/4.1	12543619	100	Lectin chain B	2.04	*I. hollandica*
	U0401	64.9/4.87	1708311	2	Stromal 70 KDa heat shock related protein, chloroplast	1.15	*S. oleracea*
	U601	64.9/4.87	1708311	2	Stromal 70 KDa heat shock related protein, chloroplast	1.11	*S. oleracea*
	U602	6.75/5.15	17224239	2	Arginine decarboxylase	1.48	*H. virginiana*
	I5401	41.9/6.23	1762912	15	Alcohol dehydrogenase B	1.11	*W. robusta*
	I6201	27.7/5.43	1389654	28	Cytosolic ascorbate peroxidase	3.15	*N. tabacum*
	I7001	36.2/5.78	53988164	12	Aldo–keto reductases	2.97	*F. ananassa*
	I5101	57.2/5.77	15240884	10	ATP binding kinase	1.5	*A. thaliana*
	I1201	18.2/5.03	51317985	18	Pathogenesis related protein (PR10)	1.15	*P. monticola*
	I8401	22.2/7.64	48375044	14	Putative mitochondrial malate dehydrogenase	1.21	*N. tabacum*
	I9801	14.1/4.74	12659208	32	Minor allergen hazelnut profilin	2.37	*C. avellana*
	I5102	93.4/8.21	15219919	8	ATP binding/carbohydrate binding kinase	2.51	*A. thaliana*
	I4001	19.8/10.51	98357561	34	High mobility group I/Y	1.57	
	I5202	19.7/10.51	1544762	18	HMG 1/Y protein	1.5	*Zea mays*
	I4602	70.6/5.6	35187004	13	Isoprene synthase	3.11	*P. montana*
Signaling and gene regulation	I6202	21.6/8.99	15235917	32	Calmodulin binding	1.38	*A. thaliana*
	I8001	18.9/4.7	12597890	36	Putative calcineurin	2.03	*O. sativa*
	I5202	56.5/9.6	433487	9	Maturase K	1.14	
	I8502	60.5/9.3	37518732	9	Maturase like protein	1.35	*C. multiflora*
Carbon metabolism	U8801	52.7/6.23	54306650	14	Rubisco large subunit	2.2	*M. kobus*
	U8802	37.8/7.8	33411902	14	Rubisco large subunit	3.82	*A. styracilfolia*
	U7201	31.9/6.0	1174745	11	isomerase chloroplast precursor	2	*S. cereale*
	U4501	39.2/5.41	1885326	23	Phosphoribulokinase	1.87	*P. sativum*
	D2301	26.8/5.01	224916	32	Photosystem II protein 33 kDa	0.405	*S. oleracea*
	D6301	35.5/5.63	30013657	37	OEE-1, 33 kDa subunit	0.305	*N. tabacum*
	D3601	48.5/8.14	12643758	28	Rubisco activase-2	0.301	*N. tabacum*
	D6703	48.5/8.14	12643758	37	Rubisco activase-2	0.165	*N. tabacum*
	I2302	35.6/5.84	131385	33	OEE-1, chloroplast precursors	4.31	*S. tuberosum*
	I4201	42.5/5.83	3738257	18	Cytosolic phosphoglycerate kinase-1	1.22	*P. nigra*
	I7502	11.1/5.87	27462753	23	Phosphoenol Pyruvate carboxylase	2.34	*L. albus*
Nucleic acid/protein regulators	D6704	76.6/8.34	29570334	49	Transcriptional adaptor	0.268	*T. monococcum*
	D0801	57.1/8.9	15239451	27	Protein phosphatase type 2A regulator	0.52	*A. thaliana*
	I7301	46.0/9.1	53791665	6	Putative WDR-13 protein	1.05	*O. sativa*
	I7401	57.1/8.9	15239451	19	Protein phosphatase type 2A regulator	2.86	*A. thaliana*
	I8501	53.9/6.76	25300595	15	PRM-1 homologue	1.07	*A. thaliana*
Energy Production	I3802	53.5/5.09	60391817	43	ATP synthase beta	1.55	*N. quadrivalvis*
	I4002	12.9/7.88	32563485	45	ATP synthase beta	2.12	*A. laevisphaera*
Growth and development	D6708	33.4/5.42	30694492	27	Unknown protein	0.488	*A. thaliana*
	I4601	18.9/11.6	52075944	30	Hypothetical protein	1.57	*O. sativa*
	I5702	59.6/6.61	48526687	9	P450	6.47	*T. ponticum*
Hypothetical protein	U4501	16.8	9755786	25	Hypothetical protein	1.87	*A. thaliana*
	U9702	4.59/11.83	15451575	59	Hypothetical protein	1.41	*O. sativa*
Unknown proteins	I5301	81.3/8.03	15238856	12	Unknown protein	1.28	*A. thaliana*
	I4001	11.5/5.06	12005190	25	Reverse transcriptase like protein	1.57	*A. quitensis*
Others	I9701	44.6/8.77	1707878	18	Amino methyl transferase mitochondrial precursor (glycine cleavage system T protein GCVT)	2.45	*S. tuberosum*

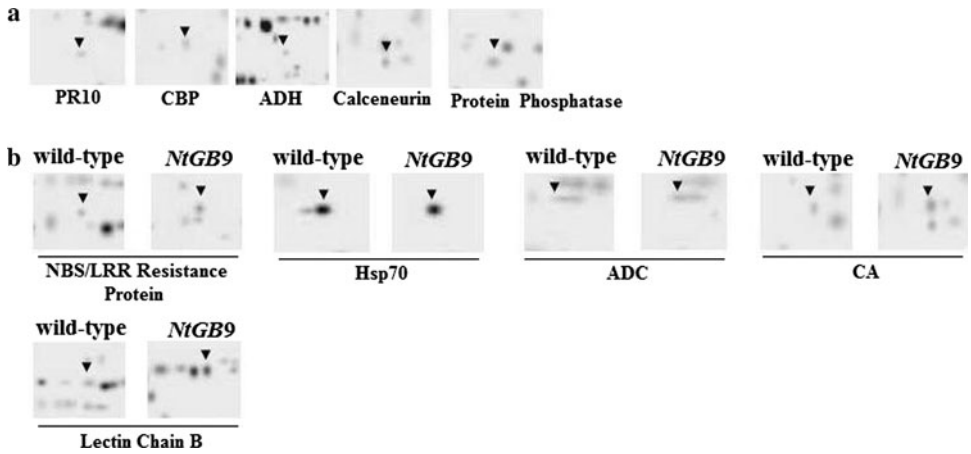


Fig. 7 Representative proteome profile of *NtGB*9 and wild type. **a** Close up of the area with spots of SA-related proteins that were induced in *NtGB* lines. **b** Close-up view of the SA-related protein spots that were upregulated in *NtGB* line in comparison with wild-type plants

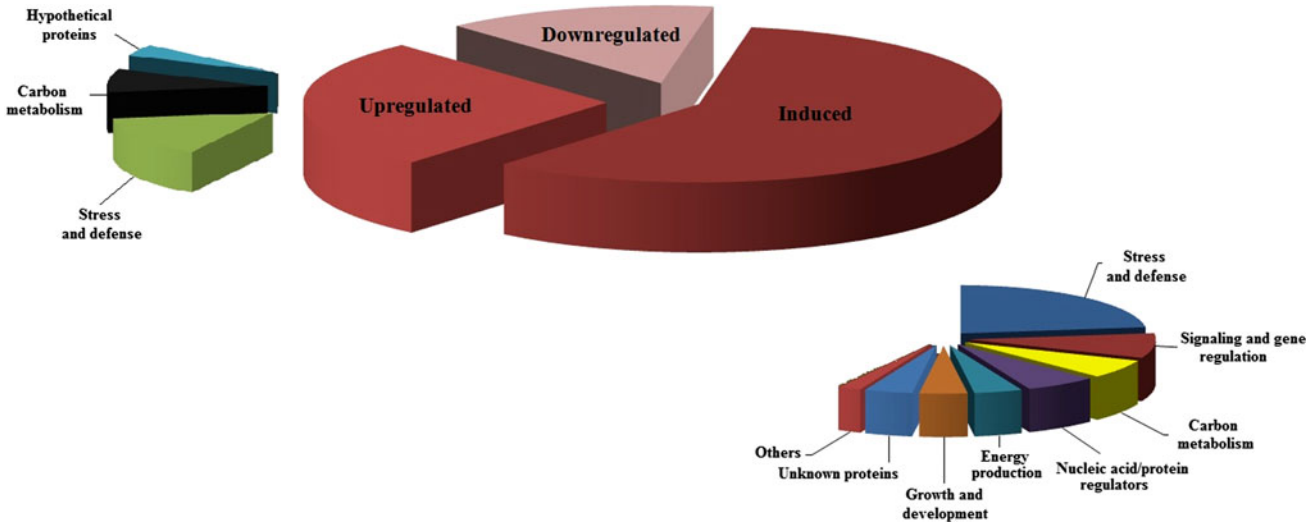


Fig. 8 Functional classification of **a** induced, **b** upregulated and **c** downregulated protein categories identified in *NtGB* lines

fungus *Cladosporium fulvum*. Enhanced resistance to *Fusarium oxysporum* due to increase in GSH level has been revealed in melon and tomato roots (Bolter et al. 1993). In another study, constitutive overexpression of SA was shown to induce GSH-mediated nickel tolerance in *Thlaspi* hyperaccumulators (Freeman et al. 2005). In this investigation, disease resistance tests revealed that *NtGB* lines showed considerable resistance to *P. syringae* pv. *tabaci;* however, in case of *A. alternata,* disease progression was observed to be almost similar to wild type. Therefore, it can be deduced that resistance of *NtGB* lines was effective against biotrophic pathogens, which involve SA (Loake and Grant 2007).

With a view to obtain further insight into the specific interactions of GSH with other established messengers, the expression profile of selected marker/pathway genes of the SA, JA and ET was investigated in transgenic T_2 generation of *NtGB* lines. This was further validated in 100 µM GSH-fed tobacco BY2 cells as well. Previous finding showed that the intensity of *NtNPR1* transcript was approximately twofold greater in the INA/SA induced samples (Cao et al. 1997). In corroboration with this, our study revealed significant upregulation of *NtNPR1* in *NtGB* lines as compared with wild type. Furthermore, onset of *NtNPR1* transcript accumulation was noted at 3 h, which was enhanced potentially up to 12 h in a time-dependent manner in GSH-fed BY2 cells. Interestingly, our results also demonstrated the enhanced expression of *NtPR1*, the marker gene of NPR1-dependent SA-mediated pathway, alongwith some other genes of the same category like *NtGLS*, *NtMAPKK*, *NtTRXh*, *NtGSNOR1* and *NtICS1*. However, *SDRLP* reported to be a NPR1-independent SA-induced gene in *Arabidopsis* (Blanco et al. 2009) showed similar level of expression to that of wild type in our study. It has been observed that during attack with *Psm*ES4326 the *PR1* expression levels in *Arabidopsis npr1-2* and *npr1-3* mutants were greatly reduced but that of *PR2* and *PR5* were not significantly affected (Glazebrook et al. 1996),

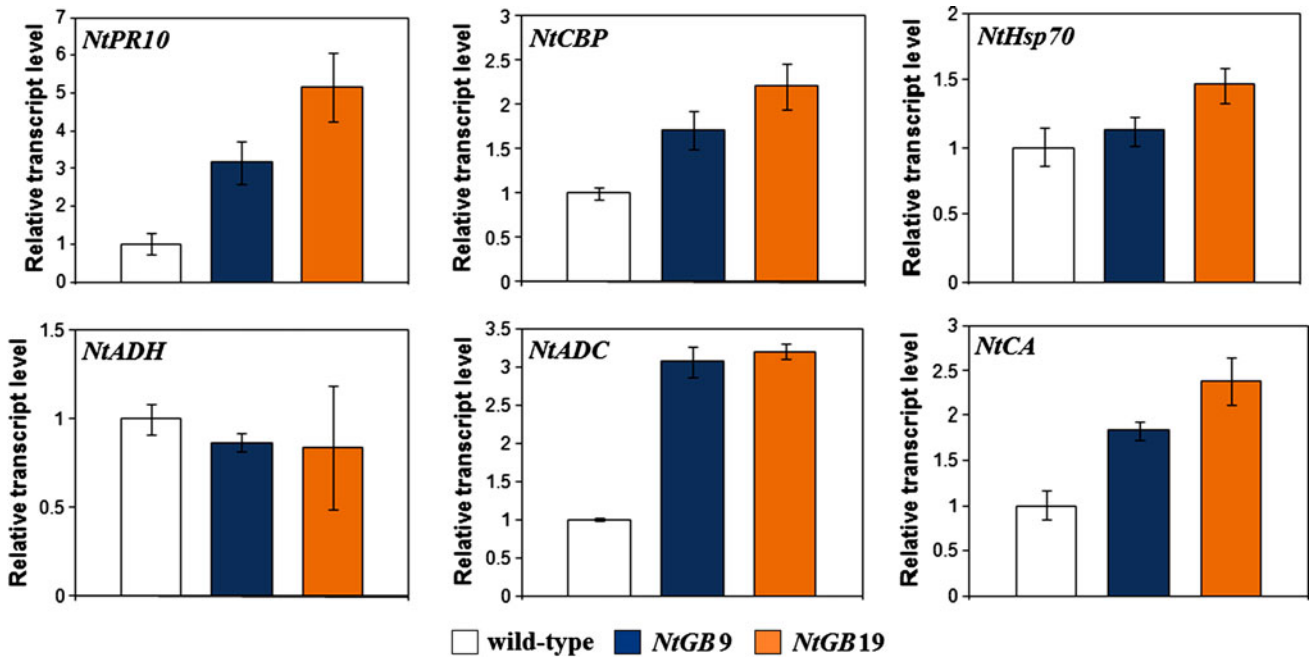


Fig. 9 Effect of *LeECS* overexpression on transcript levels of genes related to SA that were upregulated at the protein level in 2-DE in *NtGB* lines as a result of overexpression of *LeECS*. The experimental conditions were the same as in Fig. 4. Quantitative RT-PCR analyses were performed to determine the expression level of *NtPR10*, *NtCBP*, *NtHsp70*, *NtADH*, *NtADC* and *NtCA*. *Actin* was used as an internal control. Data are the mean ± SD for three individual experiments ($n = 3$) using plants grown independently

which can be correlated well with our study. Similarly in another study, the expression of NAD(P)-induced *PR2* and *PR5* in *Arabidopsis npr1-1* mutant was not affected (Zhang and Mou 2009). Similarly, the transcript levels of NPR1-independent *PR2* and *PR5* in both overexpressor and disruptant of ADP-ribose/NADH pyrophosphohydrolase were noted to be mostly similar to control plants (Ishikawa et al. 2010). However, in *Arabidopsis npr1-1* as well as in *sai1* mutant, which is allelic to *npr1,* neither *PR1* nor *PR2* and *PR5* were expressed as a result of SA treatment (Cao et al. 1994; Shah et al. 1997).

Additionally, to catch a more dynamic picture of how GSH interacts with other signaling molecules, the gene expression profiles of *NtPR1*, *NtPR2*, *NtPR4* and *NtPR5* were determined in *P. syringae* pv. *tabaci*-infected *NtGB* lines with respect to time, which eventually exhibited variable expression pattern. The increased expression profiles of *NtPR1* and *NtPR4* with time after pathogen infection can be corroborated with previous studies in *Arabidopsis* and Chinese cabbage, respectively (Wildermuth et al. 2001; Du et al. 2009; Ryang et al. 2002). However, in infected condition the change in the expression level of *NtPR2* and *NtPR5* was not that impressive. Previous reports have variable views on the coordinated regulation between *PR1*, *PR2* and *PR5*. Some studies showed that they are coordinately regulated (Uknes et al. 1992; Görlach et al. 1996; Cao et al. 1994; Delaney et al. 1995; Shah et al. 1997; Bowling et al. 1997; Clarke et al. 1998). In contrast, some other reports hinted an SA-independent *PR2*/*PR5* expression after inoculation with *P. syringae* (Glazebrook et al. 1996; Rogers and Ausubel 1997; Zhou et al. 1998; Nawrath and Métraux 1999). Further, a recent study in *Arabidopsis* demonstrated that *PR2* and *PR5* did not show any induction after *P. syringae* infection while *PR1* was strongly induced in enhanced SA condition (Beak et al. 2010). Thus, to clarify further the mode and path of action of *PR2* and *PR5*, which still is a mystery, needs further investigation.

ET is known to enhance SA/NPR1-dependent defense responses and potentiates SA/NPR1-dependent *PR1* transcription and also GSH content is known to be related in some way or the other to ET (de Vos et al. 2006; Yoshida et al. 2009). In our investigation, the expression levels of *NtPR4*, as well as *NtACCOx*, were also noted to be upregulated in *NtGB* lines. However, there was no change in expression of *NtAOS*.

It is worthwhile to mention that about 38% of induced and upregulated proteins belonged to the category of stress and defense as revealed from functional classification of the differentially expressed proteins. Many of the proteins identified here were reported previously to be induced/accumulated through SA-mediated pathway. Out of these, calcineurin, identified in our study, is a calcium sensor protein and is involved in stress and calcium signaling in plants. Transcript levels of calcineurin B-like proteins have been found to be upregulated in response to various

stresses including SA in *Pisum sativum* (Tuteja and Mahajan 2007). Most PRs and related proteins are induced through the action of the signaling compounds SA, JA or ET (van Loon et al. 2006). Here, in *NtGB* lines, the pathogenesis-related protein, PR10, was identified in induced category. PR10 is known to be induced by acibenzolar-*S*-methyl (ASM), a functional analog of SA (Ziadi et al. 2001). Calmodulin regulates SA-mediated plant immunity (Du et al. 2009). The protein identified here was calmodulin-binding protein, which enhances the ability of calmodulin to bind Ca^{2+} and activate other enzymes (Zielinski 1998). Other than these, KR4 having conserved motifs of R gene products such as NBS and LRR domains was induced by exogenous SA (Wang et al. 2004) and was also identified in our study. In addition, SA enhances heat-induced Hsp/Hsc70 accumulation in plants (Snyman and Cronjé 2008), which can be correlated with the present study. In addition, SA-induced accumulation of ADH transcripts in *Solanum tuberosum* (Matton et al. 1990) and significant increase in the expression of ADC gene as a result of SA treatment (Jang et al. 2009) were reported previously which was identified at protein level in this study. CA is the most abundant soluble zinc-containing protein in the chloroplast of plant. It has been reported earlier that tobacco chloroplast CA acts as a SA-binding protein like SABP3, which was isolated from stroma of chloroplast (Slaymaker et al. 2002) and also identified in our study. Legume lectin protein was reported as NPR1-dependent SA-binding protein (Blanco et al. 2009), which can be correlated with our data.

In conclusion, the present study demonstrates that biotic stress tolerance against biotrophic *P. syringae* pv. *tabaci* is more effective than necrotophic *A. alternata* in *NtGB* lines exhibiting enhanced GSH content. Comprehensive analysis of transcriptomic and proteomic data derived from our study suggests that GSH plays an important role to mitigate biotic stress in induced disease resistance, which probably occurs through NPR1-dependent SA-mediated pathway. This knowledge provides further insight into this expanding area on the involvement of GSH in the cross-communication with other established signaling molecules in defense response.

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Conflict of interest The authors declare that they have no conflict of interest.

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