ORIGINAL ARTICLE

AAL-toxin induced stress in *Arabidopsis thaliana* is alleviated through GSH-mediated salicylic acid and ethylene pathways

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Abstract

AAL toxin, the major virulence factor of *Alternaria alternata* f. sp. *lycopersici*, is recognized to cause necrotic cell death in plants. Glutathione (GSH) is a noteworthy participant in plant defence. However, how GSH is involved in regulating the AAL treated cell death is yet to be explored. Here, *Arabidopsis thaliana* Col-0, and previously developed transgenic line *AtECS1*, were exogenously treated with AAL toxin and a proteomic profile (ProteomeXchange accession: PXD017124) was obtained by nano LC–MS/MS analysis. Few salicylic acid (SA) and ethylene (ET) responsive proteins, along with others were identified. Selected SA-responsive genes were noted to be up regulated in AAL treated *AtECS1* compared to Col-0 by quantitative real time-PCR (qRT-PCR), beside the up regulation of *ascorbate peroxidase 1* (*APX1*) and chaperone like *heat shock protein* (*HSP*), together with *myrosinase*. Interestingly, ET biosynthetic and signaling marker genes were down regulated in AAL treated *AtECS1* compared to Col-0. Augmentation of SA content and proteins regulated by it, while, reduction of endogenous 1-aminocyclopropane-1-carboxylate (ACC) content and ET-related proteins was significant in AAL treated *AtECS1* compared to Col-0. Collectively, these findings suggested that under necrotrophic attack as mimicked here by AAL treatment, GSH may be involved in resistance primarily by SA-mediated ET suppression in addition to various stress responsive molecules.

Key message

GSH mediated resistance to AAL toxin may be conferred in *Arabidopsis* by regulating SA and ET pathways along with other stress related molecules to reduce necrotic cell death.

Keywords AAL toxin · *Arabidopsis thaliana* · Glutathione · Ethylene · Salicylic acid · Myrosinase · Hsps

Abbreviations

DAPI	4′, 6-Diamidino-2-phenylindole
PCD	Programmed cell death
PCR	Polymerase chain reaction
HPLC	High performance liquid chromatography
LOH2	LAG one homologue 2

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Introduction

AAL toxin is a type of sphinganine analogue mycotoxins (SAMs) that inhibit eukaryotic sphinganine *N*-acyltransferase (acyl-CoA-dependent ceramide synthase), which is the key enzyme in the sphingolipid ceramide biosynthetic pathway. It is the major virulence effector molecule produced by toxigenic and necrotrophic *Alternaria alternata* f. sp. *lycopersici*. Among the five series (TA, TB, TC, TD, and TE) of AAL toxin, TA is the most common kind of AAL toxin (Xu and Du 2006). AAL-toxins affect organisms other than host plants and thus their toxicity is host selective but not host specific (Tsuge et al. 2013).

Glutathione (γ-glutamylcysteinylglycine), an abundant and ubiquitous low-molecular-weight tripeptide thiol, exists in two forms viz. oxidized, GSSG and reduced, GSH. It is found in millimolar concentration in all aerobic organisms and in almost all cell organelles. It has long been known

that GSH played a significant role in plant defence (Dron et al. 1988; Wingate et al. 1988). The strategic position between oxidants viz. ROS and cellular reductants makes the GSH system perfectly configured for signaling functions, for cell cycle progression, and for regulating several epigenetic events (Foyer and Noctor 2005; Diaz Vivancos et al. 2010). Also, the role of GSH in the regulation of expression of several stress and defence genes, besides maintaining growth and development of plants is a well established fact (May et al. 1998; Ball et al. 2004). It is involved in conferring resistance against abiotic stresses like freezing, salinity stress, heavy metal tolerance, drought, detoxification of several xenobiotics, etc. (Ruiz and Blumwald 2002; Li et al. 2006; Cummins et al. 2011; Lata et al. 2011; Zagorchev et al. 2013; Gulyas et al. 2014; Sinha et al. 2015). In addition, several other evidences also reports the involvement of GSH in controlling biotic stresses induced by bacteria, fungi, and virus, by controlling oxido reduction of other thiols, by post translational modifications of protein or by inducing several plant defence genes through an intricate network of signaling molecules viz. SA, jasmonic acid (JA), ET, abscisic acid (ABA), reactive oxygen species (ROS), etc. (Parisy et al. 2007; Ghanta and Chattopadhyay 2011; Ghanta et al. 2011; Wang et al. 2011; Kuźniak et al. 2014; Cheng et al. 2015; Datta et al. 2015; Hernández et al. 2017).

Phytohormones like SA, JA, ET, ABA as well as ROS are known to play crucial roles as signaling molecules, in maintaining normal homeostasis in plants as well as participate in plant defence when threatened with a broad range of stresses. Crosstalk between these molecules has been known for decades to mitigate stress in plants (Chen et al. 1993; Thomma et al. 1998; Kunkel and Brooks 2002; Grant and Jones 2009). SA signaling is known to control the resistance to biotrophic infection, while ET and JA signaling is well-known to control necrotrophic infection in plants (Glazebrook 2005; Loake and Grant 2007). However, several studies are there where SA has been known to check the progression of necrotrophic infection in plants, as well (Murphy et al. 2000; Kouzai et al. 2018). Apart from the role of SA, ET, JA, etc. in defence response, they also play an important role in plant PCD (Van Breusegem and Dat 2006; Reinbothe et al. 2009; Wang et al. 2017). Several studies showed that JA and ET have important roles to play in AAL toxin induced PCD (Egusa et al. 2009; Zhang et al. 2016). Also, it has been found that in *Arabidopsis* LOH2 mutant, the ET pathway genes and the genes responsive to ROS were earliest to be up regulated on AAL treatment (Gechev et al. 2004). Another study showed that while, JA and ET conferred susceptibility of tomato plants to AAL, SA conferred resistance to *A. alternata* infection (Jia et al. 2013). Till now, though, evidences are there in understanding the *A. alternata* and AAL mediated necrotrophism and PCD through JA, ET,

and SA pathway, but how GSH takes part in regulating AAL mediated necrosis and PCD has been unknown till date. To elucidate, we performed an initial proteomic study with wild type Col-0 and *AtECS1*, the transgenic *A. thaliana* line exhibiting enhanced GSH content (Datta et al. 2015), with or without AAL treatment. Selected SA and ET responsive identified protein species was further analyzed by qRT-PCR and western blot. Together, GSH seems to play an important role in conferring resistance to AAL toxin, possibly by interacting through SA and ET mediated pathways, besides influencing several other effector molecules related to stress resistance.

Materials and methods

Plant growth and AAL treatment

Wild type *Arabidopsis thaliana* plants of Columbia ecotype (Col-0; Nottingham Arabidopsis Stock Centre, N1093) and transgenic line viz. *AtECS1*, developed earlier (Datta et al. 2015), were grown in Murashige and Skoog (Murashige and Skoog 1962) medium and maintained in a growth chamber at 22 ± 1 °C under a 16 h light/8 h dark cycles as standardized before (Datta et al. 2013). Leaves of four weeks old plants were treated with 10 µM AAL toxin (Chemfaces, China) with needleless syringe as described previously (Willekens et al. 1997) and maintained for 4 days (96 h). Control plants were treated with water (mock treated). After four days, samples were harvested, for all subsequent studies.

Measurement of chlorophyll content

Chlorophyll content was measured following Lichtenthaler (1987). Briefly, leaves were crushed in liquid nitrogen and chlorophyll was extracted at 95% ethanol overnight and spectrophotometric absorption of the samples was noted.

Estimation of callose deposition

Estimation of callose deposition was done following Underwood et al. (2007). Briefly, leaves were cleared of pigment by vacuum-infiltration of alcoholic lactophenol and incubated at 65 °C for 30 min. The leaves were transferred to fresh alcoholic lactophenol solution and incubated at room temperature overnight. Cleared leaves were rinsed briefly in 50% ethanol, then in water, and stained with 0.01% aniline blue (Sigma, USA) in 150 mM K_2HPO_4 (pH 9.5). The fluorescence was observed with Olympus IX 81 microscope using DAPI filter.

DAB assay

Detection of hydrogen peroxide (H_2O_2) was performed by staining leaves with 3, 3′-diaminobenzidine (DAB) (Sigma, USA) following Thordal-Christensen et al. (1997) with a slight modification. Leaves were stained in DAB solution for about 8 h. Following incubation, the DAB solution was replaced by bleaching solution (ethanol:acetic acid:glycerol = 3:1:1) and boiled for 15 min. After replacing with fresh bleaching solution, leaves were allowed to stand for 30 min. Leaves were visualized for DAB staining.

Protein extraction and digestion

Total protein from mock treated and AAL treated samples were isolated using the phenol extraction method (Isaacson et al. 2006), with minor modification. The experiment was repeated thrice for all four samples. Briefly, about 500 mg of leaf tissues from each sample were grounded 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 phenyl methyl sulfonyl fluoride). After washing the pellet and drying, it was dissolved in 8 M urea in 10 mM Tris buffer. Protein concentration was determined using Bradford reagent (Bradford 1976) using BSA as standard. Approximately 100 μg of protein from each sample were digested with trypsin (Promega Trypsin Gold MS grade, USA) with protease to substrate ratio of 1:50 for 16 h at 37 °C. The digested proteins were lyophilized and finally dissolved at 0.1% formic acid in 50% acetonitrile. The samples were then desalted using Zip-Tip μ-C18 (Millipore, USA) and used for further analysis.

Protein identification using nano LC–MS/MS

Peptides were loaded onto EASY-nLC 1000 (Thermo Scientific™, USA) where initially peptides were separated in C18 trap column (C18 3 μm, 75 μm × 20 mm), an analytical column (C18 3 μm, 75 μm × 150 mm), using mobile phase A (0.1% v/v formic acid in water) and B (0.1% v/v formic acid in acetonitrile), using the following gradient: 5–35% B for 0–100 min, 35–50% B for 100–110 min, 50–95% B for 110–115 min, 95% B for 115–120 min, 95–5% B for 120–125 min and 5% B for 125–130 min with 300 nL/min flow rate. The MS/MS analysis was conducted using LTQ orbitrap XL (Thermo Fisher Scientific, USA). A full mass spectrometry (MS) scan (350–2000 m/z) was performed at the positive ion mode with a resolution of 50,000, with an AGC value of 1*e6, maximum IT of 100 ms and dynamic exclusion of 30 s. The raw LC–MS/MS files were searched by using Mascot 2.4 (Matrix Science https://www.matrixscience.com/help/apr2012.html) containing Proteome Discoverer1.4 (Thermo Fisher Scientific, USA), against the database of *Arabidopsis thaliana* available from Swissprot. Search parameters included carbamidomethylation of cysteine as a static modification, and oxidation of methionine as dynamic modification. The proteolytic enzyme was specified as trypsin, and maximum missed cleavage of 2 was allowed. Peptide mass tolerance was set at 10 ppm and fragment mass tolerance was set at 0.8 Da. An automatic decoy database search was performed as part of the search. False discovery rates (FDR) for peptide identification were < 1.0%. To improve the accuracy and sensitivity of peptide identification Mascot results were filtered through Mascot Percolator package. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (https://www.ebi.ac.uk/pride/archive/) via the PRIDE partner repository (Vizcaíno et al. 2013) with the accession: PXD017124. Among the identified proteins, those with at least 1 unique peptide and score 30 and above, with PSM values more than 1 were considered for further studies.

Functional categorization of identified proteins was performed using KEGG (Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp/kegg/) (Kanehisa and Goto 2000). PANTHER (https://pantherdb.org/) classification tool was used to group the identified proteins into biological process, molecular function and cellular components and also under several protein classes (Mi et al. 2019).

RNA isolation and PCR

Total RNA isolation and consecutive qRT-PCR from mock treated along with AAL treated samples were performed as standardized before (Datta and Chattopadhyay 2015; Datta et al. 2015). Total RNA was extracted using TRIzol method. cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific, USA) using 1 μg of RNA from each sample and proceeded for qRT-PCR. The qRT-PCR analysis was performed using Light Cycler 96 System (Roche Applied Science, USA) with iTAQ ™ Universal SYBR® Green Supermix (BIO-RAD, USA) following manufacturer's instructions. PCR amplification was performed at 94 °C for 30 s, annealing for 15 s and 72 °C for 30 s, with a preincubation at 94 °C for 600 s. The primers used for qRT-PCR were listed in Supplemental Table S1. The constitutively expressed *Elongation factor-1alpha* (*eEF-1 alpha*) was used as the reference gene.

Western blot analysis

Western blot was done as standardized previously (Datta et al. 2015). Briefly, leaves were crushed in liquid nitrogen and proteins were extracted after homogenizing leaves in 50 mM potassium phosphate buffer, pH 7.8, containing 0.15% (v/v) Triton X-100. Protein samples were quantified by Bradford assay (Bradford 1976), resolved in 12%

SDS PAGE gel and transferred onto polyvinylidene difluoride membrane (Millipore, USA) and blocked with 5% skimmed milk. The PR4 and PR5 protein bands were detected by using a rabbit polyclonal anti-PR4 (dilution 1:2000) and anti-PR5 antibody (dilution 1:10,000) as the primary antibody and an anti-rabbit IgG conjugated to horseradish peroxidase as the secondary antibody (Agrisera, Sweden). For detecting ACO1 proteins, goat polyclonal anti-ACO1 antibody (Santacruz Biotechnology, China; dilution 1:200) were used as the primary antibody and an anti-goat IgG conjugated to horseradish peroxidase as the secondary antibody. Tubulin (Agrisera; dilution 1:1000) was used as housekeeping control. SuperSignal West Pico chemiluminescent substrate (Pierce, USA) was used for visualizing immunoreactive proteins. The experiment was repeated thrice for all four samples.

HPLC analysis

Estimation of SA content

Extraction and quantification of SA was performed following Freeman et al. (2005). SA was quantified by HPLC (Waters, USA) with a fluorescence detector by using Symmetry C18 reverse-phase column (5 μm, 4.6×250 mm) at excitation and emission wavelength of 254 nm and 395 nm respectively. 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 over 22 min.

Estimation of ACC content

Estimation of ACC was performed using o-phthaldialdehyde pre-column derivatization method as standardized before by Bushey et al. (1987) and Datta et al. (2015). The HPLC analysis was conducted using a 515 HPLC pump with a 2475 fluorescence detector as mentioned above; at a flow-rate of 0.6 mL/min. AccQ-Tag ($3.9 \times 9 \times 150$ mm) column with an excitation wavelength of 325 nm and an emission wavelength of 465 nm was used. The elution solvents were: solvent A, composed of 0.1 M sodium acetate at pH 5.8 and methanol as solvent B. Initially B was 10%. From 0 to 4 min, a linear gradient of 10–44% B was applied, from 4 to 10 min, a linear gradient of 44–50% B was applied, from 10 to 12 min a linear gradient of 50–80% B was applied, from 12 to 16 min, 80–100% B and from 16 to 20 min and finally B decreased to 0% linearly.

Statistical analysis

All experiments were repeated thrice. Mean and standard errors (SE) were calculated from three independent sets of biological replications as required. Statistical analysis was done using Student t test.

Results

Effect of AAL treatment on cell death, chlorophyll content and callose deposition in Col-0 and *AtECS1* plants

As mentioned previously (Datta et al. 2015), *Lycopersicon esculentum, γ-ECS* gene cloned in *pBI121* under CaMV35S promoter and introduced in Col-0 by *Agrobacaterium tumefaciens* mediated transformation using the floral dip method. Amongst various *AtECS* transgenic lines developed, *AtECS1* line was selected for further analysis, since this line has the significant total GSH content viz. 2.24-fold higher GSH as compared to Col-0. Interestingly, no notable morphological differences between Col-0 and transgenic *Arabidopsis* was noted. It was also found that *AtECS1* exhibited remarkable up regulation of *ACS2*, *ACS6*, and *ACO1* at transcript as well as protein levels, while they were down regulated in the GSH-depleted mutant, *phytoalexin deficient2-1* (*pad2-1*) (Datta et al. 2015).

Enhanced infectious lesions appeared in the wild type Col-0 compared to that of *AtECS1*, under AAL treated condition, in comparison to the mock treated plants (Fig. 1a–l). About 40% of the leaf area in Col-0 and 11% of leaf area in *AtECS1* were found to be damaged as found 96 h post treatment of AAL (Supplemental Fig. S1). Thus, GSH diminished the appearance of necrotic lesion in *A. thaliana* under AAL treated condition.

Leaf chlorophyll content known to be one of the key indicators of photosynthetic capacity (Cannella et al. 2016), its impairment is also an indicator of stress in plants (Carter 1994; Datta and Chattopadhyay 2015). Measurement of chlorophyll revealed a drop in its content in Col-0 plants by about 44% after AAL treatment compared to *AtECS1* where chlorophyll content dropped to 19%, after AAL toxin treatment (Supplemental Fig. S2). Total protein content was also found to be less in AAL treated Col-0 in comparison to the *AtECS1* plants by about 29% (Supplemental Fig. S3).

Leaflets were stained with aniline blue to detect callose deposition, which appeared as bright spots on blue background. The callose deposits were much more in Col-0 than in *AtECS1* after AAL treatment, which corroborated with the appearance of necrotic lesions (Fig. 2).

Study of in situ accumulation of H_2O_2 on AAL toxin treatment was done using DAB staining, where DAB is
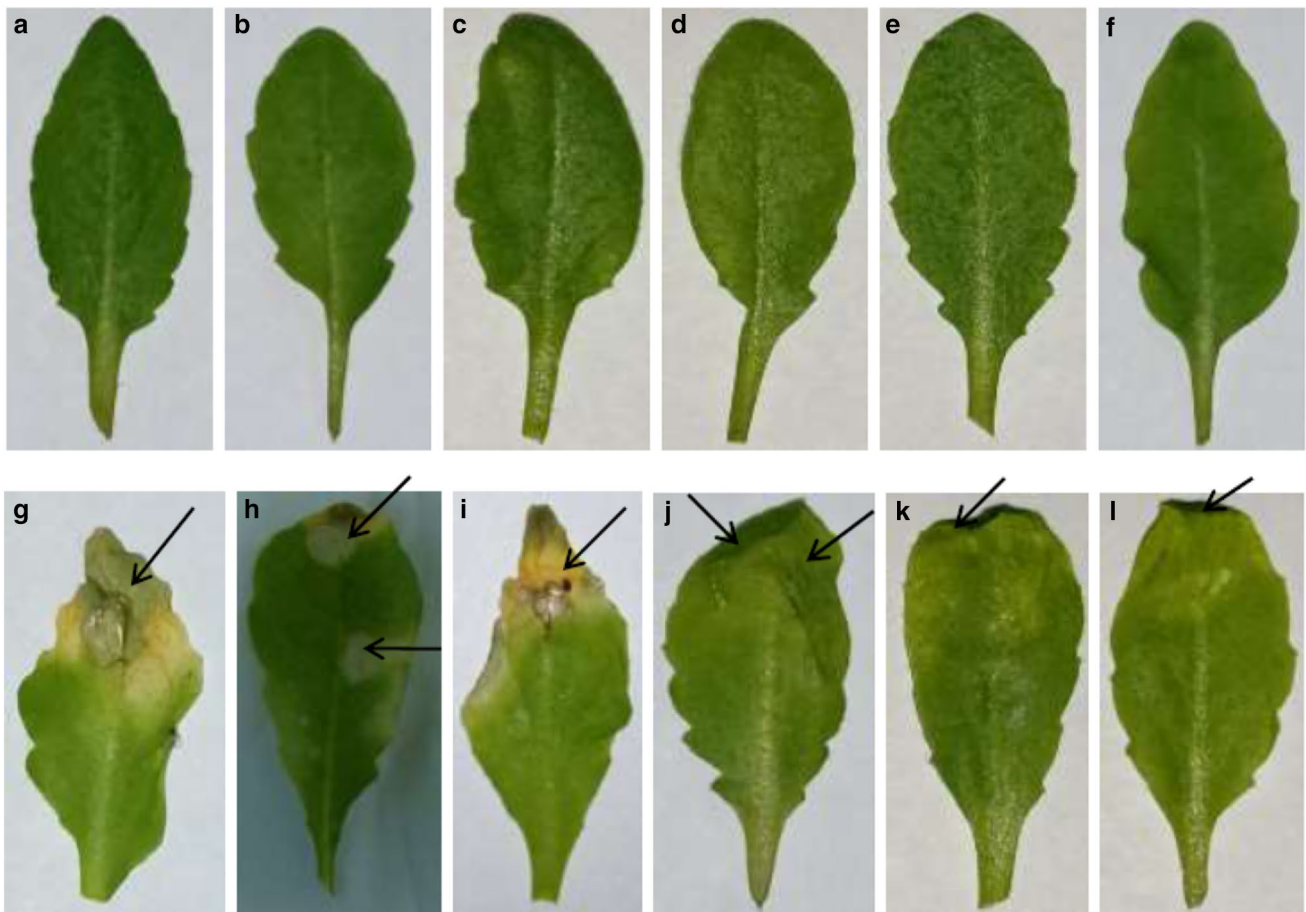
Fig. 1 Morphological study of *A. thaliana* leaves. Mock treated **a–c** Col-0 and **d–f** *AtECS1*. 10 μM of AAL treated **g–i** Col-0 and **j–l** *AtECS1*. Samples were harvested at 96 h post-treatment. Arrows indicate the infected parts

oxidized by H_2O_2 to produce a brown precipitate. More pronounced brown precipitate was noted in Col-0 plants as compared to the *AtECS1*, under AAL treated condition (Fig. 3).

Thus, GSH not only prevented loss of chlorophyll and total protein content, it also minimized the AAL induced cell death in plants as evidenced from decreased deposition of callose and H_2O_2 in AAL treated *AtECS1* compared to the wild type Col-0.

Transcript analysis of selected stress marker genes

To study the response of plants to AAL toxin and to demonstrate the establishment of infection after AAL treatment, qRT-PCR was performed at five time points like 6 h, 24 h, 48 h, 72 h and 96 h, with selected transcripts viz. *pathogenesis-related gene1* (*PR1*), *pathogenesis-related gene2* (*PR2*) and *1-aminocyclopropane-1-carboxylate synthase 2* (*ACS2*), which are stress responsive (Gechev et al. 2004; Zhang et al. 2011). Up regulation of the expression of *PR1* and *PR2*, in both AAL treated plants viz. Col-0 and

AtECS1, indicated the establishment of the infection. In case of *PR1* changes in expression between mock treated and AAL treated samples were first observed 48 h post infection, while in case of *PR2* after 24 h the changes can be observed (Fig. 4a, b). Surprisingly, though the expression of *ACS2* was up regulated in AAL treated Col-0 it was down regulated in AAL treated *AtECS1* (Fig. 4c). The change in expression of *ACS2* was found to be initiated 48 h post infection as well (Fig. 4c). *PR1* and *PR2* are established marker genes of SA-mediated defence pathway (Uknes et al. 1992; Mou et al. 2003) whereas *ACS2* is the key enzyme of ET biosynthetic pathway (Yang and Hoffman 1984). Thus GSH might be playing a role in defence against AAL toxin by interacting with SA and ET pathways. Considering the time scale experiment, it can be noted that both at morphological as well as at molecular level, significant post-treatment changes were noted at 96 h. Hence, 96 h AAL post-treatment and mock treated was selected for further analysis.
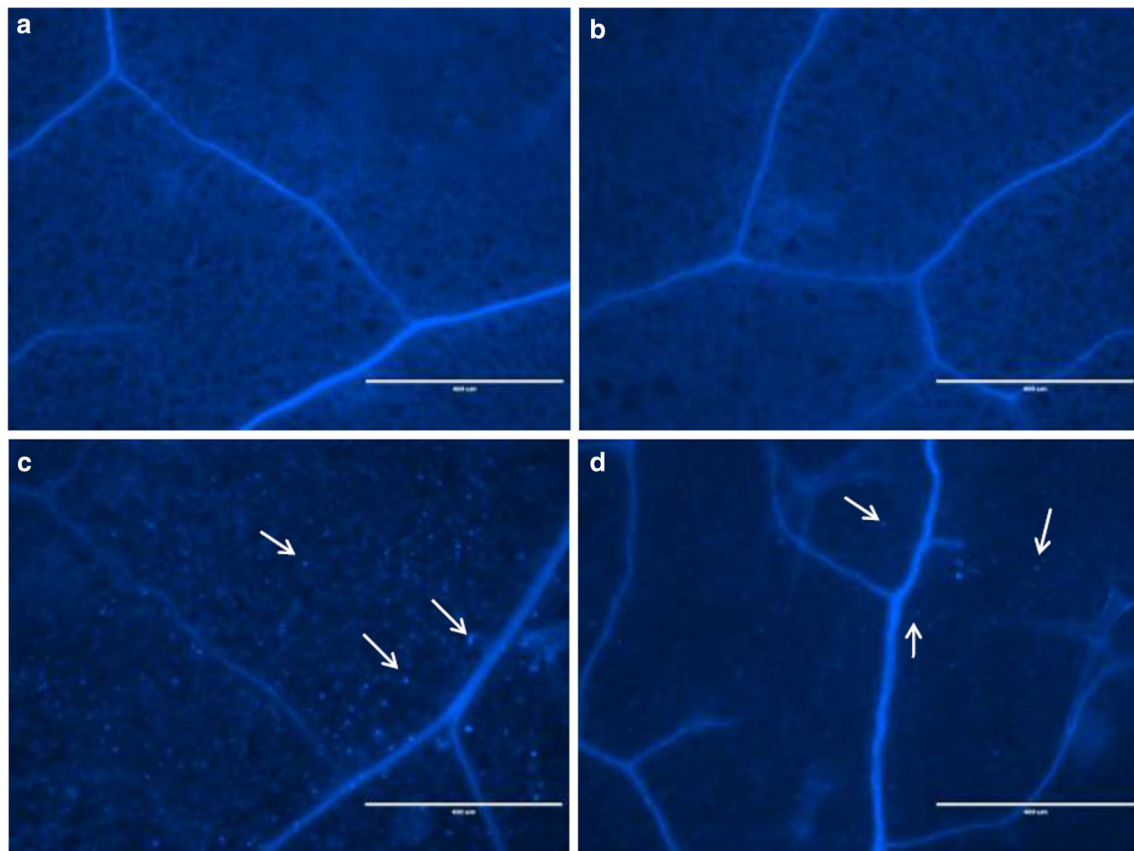
Fig. 2 Representative image of callose deposition in *A. thaliana* leaflets stained with aniline blue. In mock treated **a** Col-0 and **b** *AtECS1* and AAL treated **c** Col-0 and **d** *AtECS1*. The bright dots indicated by arrows represent callose deposition. Scale bars = 400 μm

Proteomic analysis of Col-0 and *AtECS1* under mock treated and AAL treated conditions

Proteomics approach is often undertaken to obtain a mechanistic insight to the effect of several stresses on plants (Datta and Chattopadhyay 2015; Carella et al. 2016; Huang et al. 2016). To understand the effect of GSH on AAL toxin treatment and plant defence, gel-free proteomic study was performed with three biological replicates of mock treated and AAL treated samples of Col-0 and *AtECS1,* the transgenic line. A total of 263 proteins were identified in mock treated Col-0, 269 proteins in mock treated *AtECS1*, 251 proteins in AAL treated Col-0 and 298 in AAL treated *AtECS1*, having scores above 30 and with at least 1 unique peptide count. Among them proteins having PSM values more than 1 were represented in tables (Supplemental Table S2-S5).

Functional categorization of proteins

Using PANTHER, total proteins from all four samples were classified under: biological process, molecular function and cellular component. They were also assigned under several protein classes. Although no significant changes were observed in representative proteins in Col-0 mock treated and AAL treated samples, but proteins were to some extent over-represented in AAL treated *AtECS1* samples, under all four categories viz. biological process, molecular function cellular component, and protein classes compared to other samples (Supplemental Fig. S4).

Functional categorization of the identified proteins in mock treated Col-0 showed that about 34% of the proteins were under stress and defence category, 37% of the proteins belonged to photosynthetic, respiratory, carbon and energy metabolism category and 29% of the remaining proteins belonged to other categories viz. protein and nucleotide metabolism, signaling pathways, nitrogen metabolism. In AAL treated Col-0 samples, about 35% proteins were under stress and defence category, 34% belonged to photosynthetic, respiratory; carbon and energy metabolism and 31% belonged to protein metabolism, developmental process, signaling pathways, nutrient metabolism. In mock treated *AtECS1*, about 32% were under stress and defence category, 33% under photosynthetic, respiratory, carbon and energy metabolism and 35% to other categories including nucleotide and protein metabolism, signaling pathways, amino acid metabolism. In AAL treated *AtECS1* about 38% of proteins
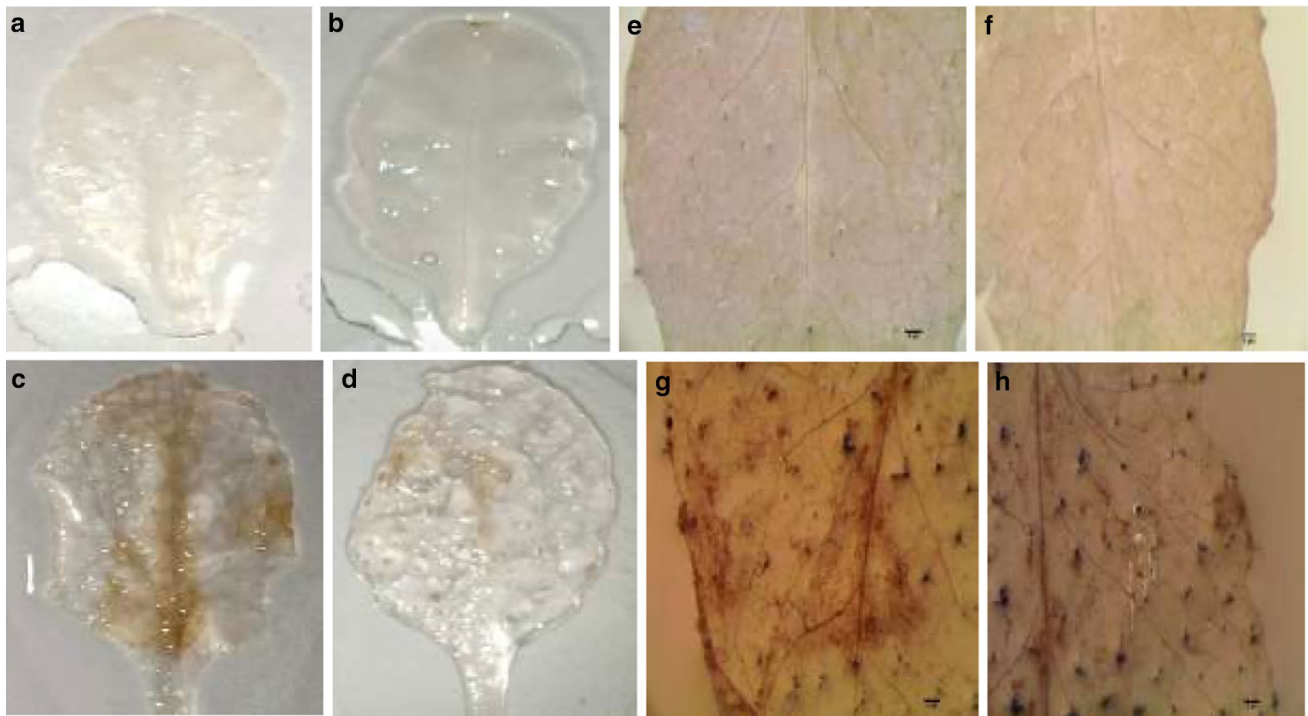
Fig. 3 Representative image of the leaves of *A. thaliana* stained with DAB. In mock treated **a**, **e** Col-0 and **b**, **f** *AtECS1* as well as in AAL treated **c**, **g** Col-0 and **d**, **h** *AtECS1*. Scale bars (in **e**–**h**) = 5 μm

were under stress and defence category, 34% under photosynthetic, respiratory, carbon and energy metabolism and 28% under protein and nucleotide metabolism, signaling, lipid metabolism, amino acid metabolism category (Fig. 5 and Supplemental Tables S2–S5). Under stress and defence category, proteins like, glutathione-S-transferases, superoxide dismutases, catalases, peroxiredoxins, APX, HSPs, myrosinases, PR 2, 4, 5 were identified. Under photosynthetic, respiratory, carbon and energy metabolism category of proteins, ribulose bisphosphate carboxylase large and small chains, oxygen evolving enhancer proteins, RUBISCO activase, chlorophyll a/b binding proteins, glyceraldehyde-3-phosphatedehydrogenase, malate dehydrogenase, phosphoglycerate kinase, sedoheptulose 1,7 bisphosphatase, ATP synthase subunit alpha, beta, etc. were identified. Several ribosomal proteins, ribosome recycling factors involved in translation were identified. Also, proteins involved in lipid metabolism viz. non-specific lipid transfer proteins, signaling pathway proteins viz. calmodulins, cytoskeletal proteins like actin were identified. Proteins related to cell death process viz. several proteins belonging to HSP 70 family, cysteine proteinase, cytochrome c were also among the identified proteins. Among these identified proteins several stress responsive proteins viz. PR2, 5, carbonic anhydrase (CA), glutathione S-transferase 8, glutamine synthetase, peptidyl-prolyl *cis*–*trans* isomerase, cytochrome b6-f complex iron-sulfur subunit, were known to be effected by SA

as well (Uknes et al. 1992; Cao et al. 1994; Slaymaker et al. 2002; Blanco et al. 2009; Zhang et al. 2016), while PR4 was also identified which was known to be ET responsive (Potter et al. 1993). A few from the above mentioned proteins were chosen for further studies to establish the role of GSH in mitigating AAL mediated stress in plants.

Expression analysis of selected stress related genes identified in proteomics

The expression analysis of some ET and SA responsive genes viz. *PR4* (Uni Prot ID-P43082; Gene ID: 819632), *PR5* (Uni Prot ID- P28493; Gene ID: 843842), *CA1* (Uni Prot ID- P27140; Gene ID: 821134), was performed by qRT-PCR, which were identified in our proteomics data. The expression analysis of ET biosynthetic gene *ACC oxidase* (*ACO1*) (Yang and Hoffman 1984) was also done to see if ET biosynthesis can get influenced due to the toxin treatment. The expressions of the above mentioned genes were up regulated in AAL treated *AtECS1* compared to AAL treated Col-0, except for *PR4* and *ACO1*, which showed lower expression in *AtECS1* compared to Col-0 after treatment with AAL. The expression of SA responsive *PR1*, were also noted as it was a strong marker of SA signaling, which got up regulated in *AtECS1* compared to the Col-0, under AAL treated condition. Thus, there is an indication of the involvement of SA and ET in defence against AAL toxin.

Fig. 4 Relative expression levels of three transcripts (**a**–**c**) at 6 h, 24 h, 48 h, 72 h and 96 h. Experiment was performed with both the mock and AAL treated Col-0 (Col C and Col T) and *AtECS1* (At C and At T) to see establishment of infection. *eEF-1 alpha* was used as an internal control. Data are the mean ± SE for 3 individual experiments (n = 3) using 3 biological replicates independently. Stars indicate significant differences between samples at *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 according to the Student t-test
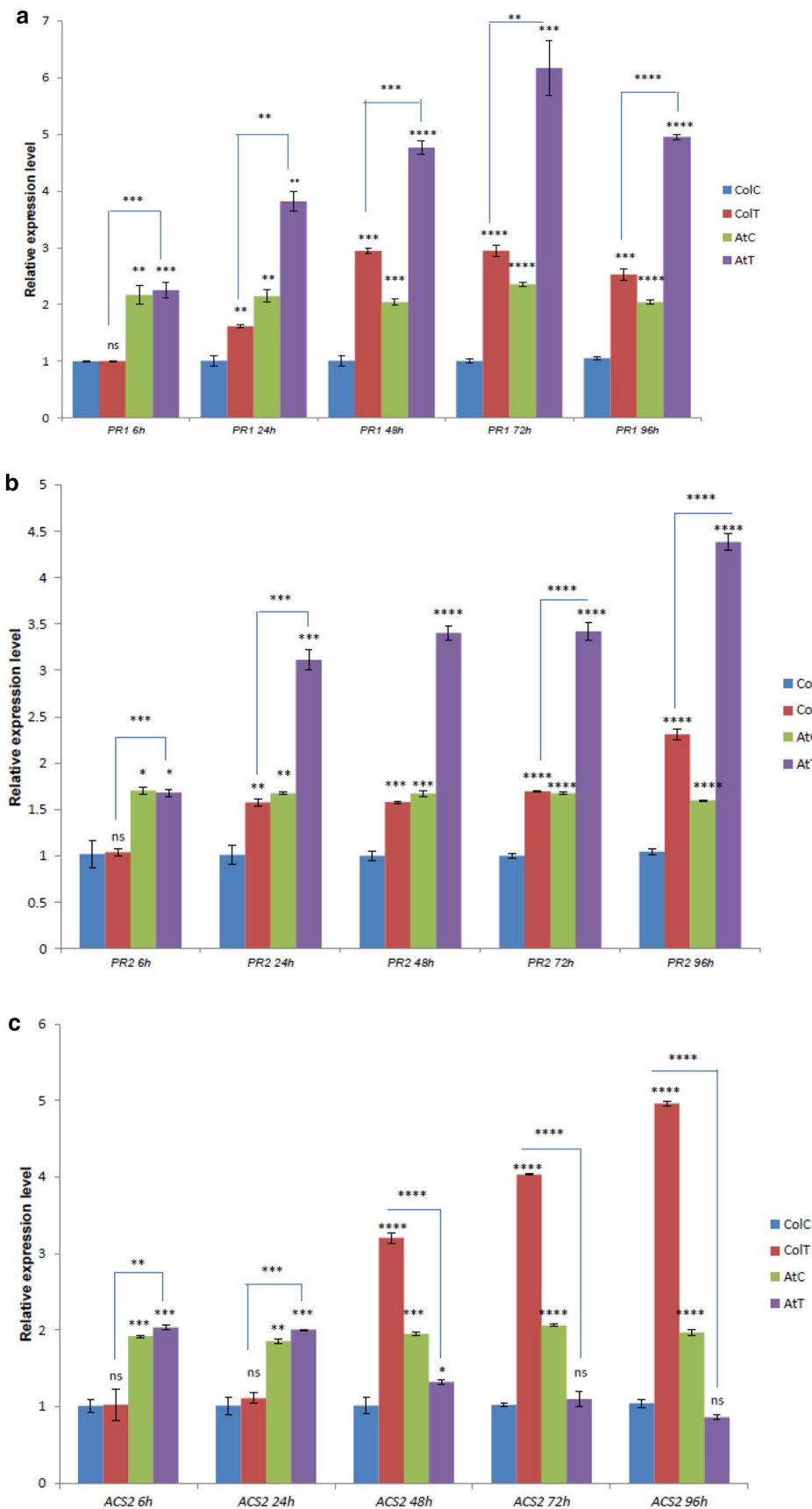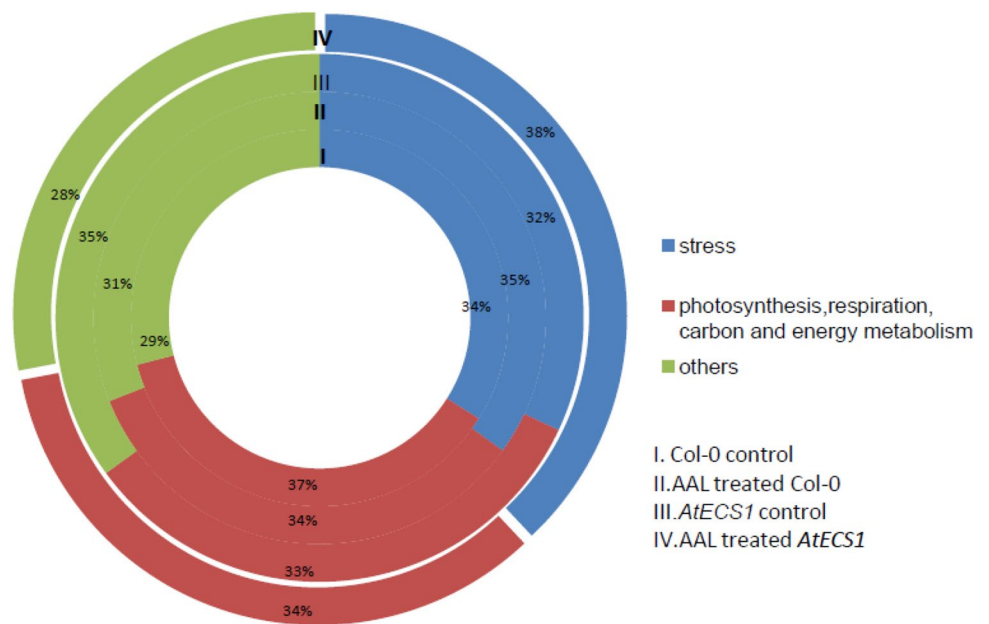
Fig. 5 Functional categorization of identified proteins in orbitrap in each sample of Col-0 and *AtECS1* under mock and AAL treated conditions

Higher expressions of *APX1* (Uni Prot ID-Q05431; Gene ID: 837304), *myrosinase* (*TGG1*) (Uni Prot ID- P37702; Gene ID: 832669) and *HSP70-1* (Uni Prot ID- P22953; Gene ID: 831020) were noted as well, in *AtECS1* compared to Col-0 under AAL treated condition (Fig. 6).

Western blot analysis of selected stress responsive protein species identified in proteomics

To check if there were any changes in the expression, even at protein level, in addition to transcripts, western blot analysis was performed, where differential accumulation of PR5, PR4 and ACO1 were found in Col-0 and *AtECS1*. PR5 was up accumulated in AAL treated *AtECS1* compared to AAL

treated Col-0, while PR4 and ACO1, were down accumulated in AAL treated *AtECS1* compared to AAL treated Col-0, though the levels of all proteins were higher in mock treated *AtECS1* compared to Col-0 (Fig. 7).

Estimation of SA and ACC content in Col-0 and *AtECS1* plants after AAL toxin treatment

To investigate if there was any direct effect of AAL toxin on the metabolite levels of SA and ACC, under differential GSH content, their estimation was performed by HPLC. It was noted that under AAL treated condition, the SA content was about 4 times higher in *AtECS1* (Supplemental Fig. S5), compared to Col-0. However, ACC content was enhanced

Fig. 6 Relative expression levels of transcripts. Experiment was performed in mock and AAL treated Col-0 (Col C and Col T) and *AtECS1* (At C and At T). *eEF-1 alpha* was used as an internal control. Data are the mean ± SE for 3 individual experiments (n = 3) using 3 biological replicates independently. Stars indicate significant differences between samples at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$ according to the Student t-test
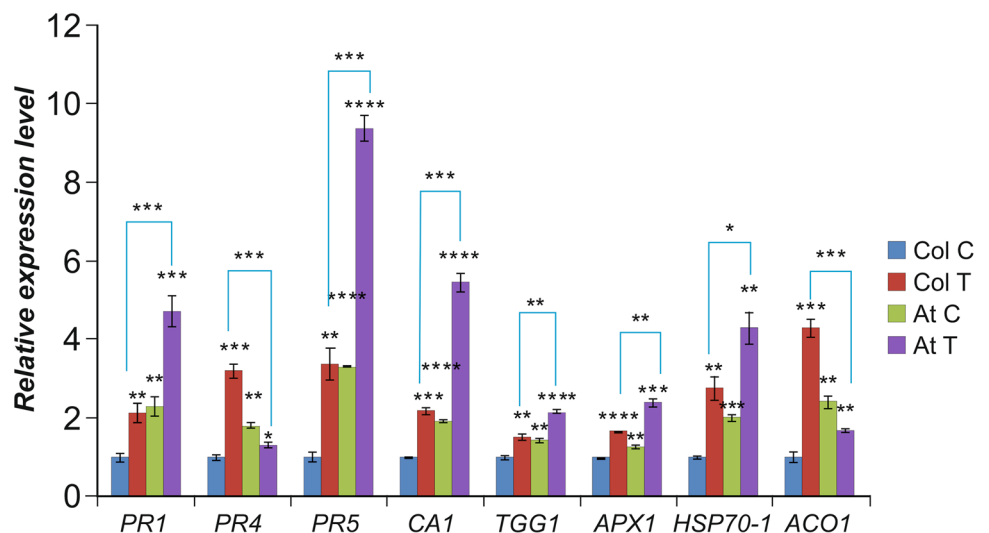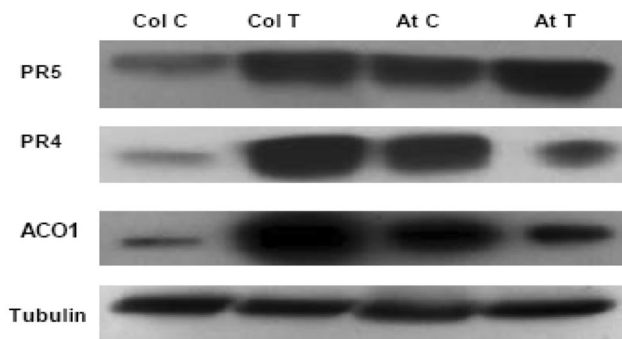
Fig. 7 Representative image of western blot analysis. Blots showing the levels of selected proteins in mock treated and AAL treated Col-0 (Col C and Col T) and *AtECS1* (At C and At T). The experiment was repeated with 3 biological replicates of all samples, independently

in Col-0 compared to *AtECS1* under AAL treated condition, which corroborated with our previous transcript data and western blot analysis (Supplemental Fig. S6).

Discussion

GSH ameliorated AAL induced necrosis and cell death in plants

Enhanced GSH in *AtECS1* caused decreased infectious lesion formation and its spreading, compared to Col-0 under AAL treated condition, as visualized in our morphological data. Since necrotrophic fungus kills the host tissues and feeds on them, it is advantageous for the host plants if there is a drop in infectious lesion formation and necrosis. Besides, presence of GSH also resulted in lower chlorophyll loss in leaves, of AAL treated *AtECS1*. Hence GSH improved the photosynthetic capacity of the plants even under stressed condition by maintaining the chlorophyll content, since photosynthetic rate and chlorophyll content are directly correlated (Fleischer 1935). This might help the plants in production of more carbon sources, which in turn helps in retaining the required biomass of plants, as well as provide the essential energy requirement for downstream defence reactions, since induction of host defence response is cost intensive (Swarbrick et al. 2006).

More pronounced degradation of protein was found in Col-0 compared to *AtECS1* under AAL treated condition. Previous study reported that protein degradation is accelerated under carbon starvation to provide an alternative source of carbon for respiration (Araújo et al. 2011). Protein degradation may also increase under several pathogen induced stresses (Beers et al. 2000) and there was evidence as well for disruption of protein biosynthetic machinery under *A. alternata* infection (Sinha and Chattopadhyay 2011). In *AtECS1* higher amount of chlorophyll, which indicated

higher photosynthetic rate, could have compensated for the carbon starvation, and hence lowering protein degradation for respiration, under AAL treated condition. Moreover, GSH can help in lowering degradation of protein or protect them from getting damaged by activating other intrinsic defence mechanism (Park and Seo 2015; Kumar and Chattopadhyay 2018), under AAL treated condition.

Both callose deposition and the outburst of H_2O_2, which measures the extent of cell death, showed higher accumulation in Col-0 than in *AtECS1* under AAL treated condition. H_2O_2 has been known as a signaling molecule which can help with stress acclimation, when present in low concentration, but also known to trigger cell death when present in excess amount (Gechev et al. 2002; Dat et al. 2003; Apel and Hirt 2004). Its overproduction can be a strategy to kill the host tissue in the initial phase of infection, in case of necrotrophic fungus (Tiedemann 1997). Previously it was known that *A. alternata* and AAL toxin as well, caused an oxidative outburst and concomitant deposition of callose in several plants including *Arabidopsis* (Gechev et al. 2004; Zhang et al. 2011; Chung 2012). GSH has been widely known for its antioxidative effect and as a scavenger of ROS molecules (Noctor and Foyer 1998). *AtECS1* thus have gained an advantage over Col-0 in suppressing ROS induced cell death, triggered by AAL toxin.

Insight into GSH-mediated molecular changes to alleviate AAL induced stress in plants

The role of GSH in conferring stress tolerance in plants has been known. While external feeding of GSH or overexpressing lines having higher GSH content were always found to be more resistant to several stresses compared to wild type plants (Ghanta et al. 2011; Datta et al. 2015; Sinha et al. 2015), GSH depleted plants display susceptibilities to range of stresses (Dubreuil-Maurizi et al. 2011; Sobrino-Plata et al. 2014; Datta and Chattopadhyay 2015; Kumar et al. 2015). The resistance or susceptibilities of plants are direct manifestations of molecular changes happening inside the plants in response to stress. Proteomics studies revealed that while plants displaying higher GSH contents manifested enhanced expressions of stress and defence related proteins (Ghanta et al. 2011; Datta et al. 2015), a range of defence related proteins were down regulated in GSH depleted mutant *pad2.1* (Datta and Chattopadhyay 2015; Kumar et al. 2015). Investigations on GSH depleted mutant viz. *pad2.1* has been reported previously either in absence (Datta et al. 2015) or presence of both biotic/abiotic stresses (Datta and Chattopadhyay 2015; Kumar et al. 2015) and identified several down regulated defence related proteins. Considering this, presently we performed proteomic profiling of the transgenic line *AtECS1* exhibiting enhanced GSH content to

understand the mechanistic interaction of GSH with other molecules in mitigating fungal toxin induced stress in plants.

To investigate the role of GSH in plant defence against AAL toxin the initial proteomic analysis that was performed identified proteins under several categories. Among them, few proteins were found which were reported to be related to cell death in plants. PCD is controlled sequential events that lead to cell death (Lockshin and Zakeri 2004). It is crucial for multicellular development as well as in defence response by restricting the pathogens (Lam 2004). Fungal AAL toxin is known as one of the potent inducers of plant cell death (Wang et al. 1996). In the present proteomic study cysteine proteinase RD21A has been identified. It has been known to be involved in cell death in the transmitting tract and septum epidermis during flower development (Boex-Fontvieille et al. 2015). The protein has been known to function as a PCD promoting protease during infection by the necrotrophic fungal pathogen *Botrytis cinerea* and may participate in cell death in stressed or injured cells (Hayashi et al. 2001; Lampl et al. 2013). The protein was also found to be up regulated in tomato treated with AAL toxin (Zhang et al. 2016). Cytochrome C-1 has also been identified in our proteomics data. The release of cytochrome c from mitochondria is known to be an early event related to plant PCD (Balk et al. 1999; Balk and Leaver 2001). It has been reported to interact with a range of targets on PCD (Martínez-Fábregas et al. 2013). Along with the above mentioned proteins several members of HSP family proteins like, HSP70-1, HSP70-3, HSP70-2, HSP70-11 has also been identified in our proteomics data. While study reported that HSP can contribute to PCD during re-modelling of lace plant leaves (Rowarth et al. 2019) another report emphasized the role of HSP in inducing apoptosis in response to necrotrophic pathogen (Byth-Illing and Bornman 2014). Another study with tobacco protoplasts reported that HSP70 might play a role in suppression of apoptosis under increased temperature, during pathogen infection (Cronjé et al. 2004).

A set of proteins were SA and ET responsive. Previous comparative proteomic study in our lab between wild type and transgenic mint over expressing γ-ECS, having enhanced GSH content under *A. alternata* infection, identified differentially expressed proteins (Sinha et al. 2013). Corroborating with this and other evidences (Gechev et al. 2004; Wittstock et al. 2016) a few proteins from our LC–MS/MS proteome study were chosen for further downstream studies, to establish the relation of GSH with other molecules, under AAL treated condition:

GSH acts through SA and ET pathway to diminish the effect of AAL toxin in plants

Up regulation of *PR4*, the marker of ET signaling not only occurs under ET treatment but even under AAL toxin treatment as well (Gechev et al. 2004). In our proteomics data, hevein-like protein has been identified which corresponds to *PR4* gene (Gene ID: 819632) was up regulated in AAL treated Col-0, which demonstrated that ET played an important role in AAL triggered response in plants. But, in case of AAL treated *AtECS1* the expression of *PR4* was lower than AAL treated Col-0, although the basal level of *PR4* was higher in *AtECS1* compared to Col-0, in mock treated plants. To study further the interaction of GSH and ET, in presence of AAL toxin, *ACO1* gene expression was checked. Though basal level of *ACO1* was greater in *AtECS1* compared to Col-0, which led to the increased ET content in *AtECS1*, corroborating with the previous study (Datta et al. 2015), yet the expression profile of *ACO1* was similar to *PR4* under AAL treated condition i.e. higher in Col-0 than *AtECS1*. Also, our qRT-PCR data of *ACS2* showed the same expression changes like *PR4* and *ACO1*. The western blot analysis of both PR4 and ACO1 supported the transcript data. HPLC analysis of the ACC content showed that although basal level of ACC was higher in *AtECS1* compared to Col-0, yet after AAL treatment the ACC content was much higher in Col-0 compared to *AtECS1* corroborating with the transcript and protein levels of PR4 and ACO1. There are several reports that showed ET enhances cell death and necrosis in tomato, tobacco and pears under *A. alternata* infection or AAL treatment (Moore et al. 1999; Mase et al. 2012; Wang et al. 2017). Later in *Arabidopsis* it was also reported that application of ET inhibitor AVG reduced cell death by about 50% under AAL treated condition (Gechev et al. 2004). Thus, ET potentiates the establishment of the necrotic cell death in plants, under AAL treated condition.

Again, pathogenesis-related 5 protein or PR5 has been identified here which corresponds to *PR5* gene (Gene ID: 843842), which is SA responsive gene, was found to be up regulated in AAL treated *AtECS1* compared to Col-0. The expression of SA responsive *PR1* gene also showed similar expression pattern as *PR5*. The western blot analysis of PR5 supported the transcript data as well. Since PR5 and PR1 both are SA responsive, their elevated expressions under enhanced GSH condition in *AtECS1* indicated that GSH might had led to higher SA content. HPLC analysis showed that under AAL treated condition there was change in SA content. The basal level of SA was higher in *AtECS1* as compared to Col-0. In our previous study, we also demonstrated that enhanced GSH level caused up regulation of SA content in plants under control condition (Ghanta et al. 2011). However, there was about fourfold more SA content in *AtECS1* than in Col-0, under AAL treated condition. Previous reports in tomato showed that exogenous application of SA caused to acquire enhanced resistance against *A. alternata* (Esmailzadeh et al. 2008). In *Arabidopsis* also, microarray study after AAL treatment resulted in up regulation of SA pathway genes, *PR5* and *PR1* (Gechev et al.

2004), which implied involvement of SA in regulating AAL-induced stress in *Arabidopsis*.

ET was known to induce oxidative stress and ROS also can induce ET production through modulating expressions of the two ET biosynthetic pathway enzymes, ACC synthase and ACC oxidase (de Jong et al. 2002; Wang et al. 2002). Previous study showed that in absence of AAL, exogenous addition of ACC did increase the level of ET, but didn't cause necrosis to appear (Moussatos et al. 1994). Thus, increase in ET is not the cause of necrosis and cell death, rather the presence of AAL caused a trigger of complex signaling that increased the biosynthesis and evolution of ET as well as ROS at toxic levels in the plants to cause the necrotic symptoms and cell death. So suppression of their level can be advantageous to plants, to arrest the effect of AAL toxin. Earlier study showed that exogenous application of SA caused suppression of ET pathway and hence decreased AAL mediated cell death (Jia et al. 2013). In present study, though SA level was found to be high after AAL treatment in Col-0, yet ET level was higher there as well and hence necrotic cell death was more there when compared to *AtECS1* under AAL treated condition. Therefore, a threshold level of endogenous SA is required to be crossed, to transcend the toxic effect of ET presumably by suppressing its synthesis as well as its signaling, under AAL treated condition, as noted in *AtECS1* with higher content of GSH. Thus, enhanced GSH caused SA mediated resistance by suppressing ET, when there is successful AAL mediated stress induction in plants.

GSH acts through various stress-responsive molecules to mitigate AAL induced stress

The role of CA in C3 plants had been known for decades in the conversion of HCO^{3-} to CO_2 to ensure maximum rates of fixation by Rubisco (Everson 1970; Poincelot 1972; Werdan and Heldt 1972). Besides this the role of CA as a salicylate binding antioxidant molecule has already been established (Slaymaker et al. 2002). In our present study we find that the beta carbonic anhydrase 1 protein was identified, which corresponds to *CA1* (Gene ID: 821134) and the later showed higher expression in AAL treated *AtECS1* compared to AAL treated Col-0. Thus in *AtECS1*, GSH can play a role in conferring resistance against AAL mediated stress, through up regulating CA, which not only helped in enhanced carbon fixation, but also plays a protective role against oxidative stress induced damage caused by the toxin.

Myrosinases (β-thioglucoside glucohydrolase, TGG) are the group of enzymes that cleave glucosinolates, which are amino acid-derived secondary metabolites (Barth and Jander 2006). In cruciferous plants like *Arabidopsis*, myrosinase-glucosinolate system forms a preformed chemical defence system against several insect, pathogens and herbivores

(Bones and Rossiter 1996; Raybould and Moyes 2001). It was also reported that TGG-dependent hydrolysis of glucosinolate plays an important role in the response of *Arabidopsis* to Fumonisin B (FB1), a sphinganine analogue mycotoxin like AAL toxin (Abbas et al. 1994), since lack of TGG rendered plants more sensitive to FB1 (Zhao et al. 2015). Our proteomics data identified the protein myrosinase 1 which corresponds to *TGG1* (Gene ID: 832669). Here, basal level expression of *TGG1*, was found to be higher in *AtECS1* than Col-0, and it was highly induced in *AtECS1* compared to Col-0 after AAL treatment. Thus, enhanced GSH level is capable to reduce AAL toxin mediated stress in *AtECS1* by increasing myrosinase amount and hence catalyzing glucosinolate breakdown to pathotoxic products making the plants tolerant to the toxin.

One of the causal reasons of AAL toxin and *A. alternata* mediated cell death is ROS accumulation (Gechev et al. 2004; Prasad and Upadhyay 2010). The cytosolic L-ascorbate peroxidase 1 which has been identified in our proteomics data corresponds to *APX1* (Gene ID: 837304), the later being found to give increased expression in AAL treated *AtECS1* compared to Col-0. Increasing the expression of APX, a ROS scavenging antioxidant enzyme, may be a possible way by which GSH facilitated the reduction of toxic effect of ROS and hence necrotic cell death, under AAL mediated stress. This was in agreement with previous study with *A. alternata* infected plants (Sinha et al. 2013).

HSPs are a subset of proteins, acting as the molecular chaperones and known for their rapid induction under large numbers of stresses (Wang et al. 2004; Scarpeci et al. 2008). Lower expression of HSPs can cause increased susceptibility of plants to *A. alternata* induced stress (Zhu et al. 2017). Earlier it has been reported that GSH induced the expression of several HSPs (Kumar and Chattopadhyay 2018). In present proteomic study HSP70-1 protein was identified which corresponds to *HSP70-1* gene (Gene ID: 831020). *HSP70-1* gene was found to be up accumulated in *AtECS1* compared to Col-0 under AAL treated condition, which corroborated with previous study where HSP was up regulated upon *A. alternata* infection (Sinha et al. 2013). Thus, enhanced GSH makes the plants more resistant, through the enhanced expression of HSPs.

Conclusions

In this study, 10 μM of AAL toxin was applied to the leaves of *Arabidopsis*, to establish the stress successfully. ET and ROS are the two key mediators in *A. alternata* as well as AAL induced cell death. GSH promoted mitigation of AAL induced stress via SA mediated suppression of ET. Moreover, being an antioxidant molecule itself, GSH was also found to act along with CA and APX as well to minimize

the effect of toxic ROS that is a causal agent of necrotic cell death in AAL treated plants. GSH also can act through myrosinases in addition to chaperones like HSPs, to subside the effect of the toxin in plants as well. Thus our study with the model plant *Arabidopsis* suggests that GSH can act as a potential candidate in alleviating AAL induced distress in plants by acting through several stress and defence related molecules. This might help for future research on GSH mediated mitigation of environmental stress in economically important plants.

Author contributions AS and SC designed the experiments. AS carried out the experimental and analytical works and drafted the manuscript. PB and KM helped in drafting the manuscripts and also in data analysis. SC supervised the analysis and prepared the final manuscript.

Compliance with ethical standards

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

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