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Plant responses to fungal volatiles involve global posttranslational thiol redox proteome changes that affect photosynthesis

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Abstract

Microorganisms produce volatile compounds (VCs) that promote plant growth and photosynthesis through complex mechanisms involving cytokinin (CK) and abscisic acid (ABA). We hypothesized that plants' responses to microbial VCs involve post-translational modifications of the thiol redox proteome through action of plastidial NADPH-dependent thioredoxin reductase C (NTRC), which regulates chloroplast redox status via its functional relationship with 2-Cys peroxiredoxins. To test this hypothesis, we analysed developmental, metabolic, hormonal, genetic, and redox proteomic responses of wild-type (WT) plants and a NTRC knockout mutant (*ntrc*) to VCs emitted by the phytopathogen *Alternaria alternata*. Fungal VC-promoted growth, changes in root architecture, shifts in expression of VC-responsive CK- and ABA-regulated genes, and increases in photosynthetic capacity were substantially weaker in *ntrc* plants than in WT plants. As in WT plants, fungal VCs strongly promoted growth, chlorophyll accumulation, and photosynthesis in *ntrc–Δ2cp* plants with reduced 2-Cys peroxiredoxin expression. OxiTRAQ-based quantitative and site-specific redox proteomic analyses revealed that VCs promote global reduction of the thiol redox proteome (especially of photosynthesis-related proteins) of WT leaves but its oxidation in *ntrc* leaves. Our findings show that NTRC is an important mediator of plant responses to microbial VCs through mechanisms involving global thiol redox proteome changes that affect photosynthesis.

K. A. and M. B. contributed equally to this work.

KEYWORDS

growth promotion, hormone signalling, microbial volatile compounds, photosynthesis, plant–microbe interactions, redox proteomics

1 | INTRODUCTION

It is well known that volatile compounds (VCs) emitted by beneficial rhizosphere bacteria and fungi can promote plant growth (Hung, Lee, & Bennett, 2013; Kanchiswamy, Malnoy, & Maffei, 2015; Piechulla, Lemfack, & Kai, 2017; Ryu et al., 2003) and root developmental changes (Delaplace et al., 2015; Ditengou et al., 2015; Garnica-Vergara et al., 2016; Gutierrez-Luna et al., 2010). However, recent studies have shown that pathogens may also release growth-promoting VCs (Sánchez-López, Baslam, et al., 2016). Promotion of growth and developmental changes by microbial VCs has frequently been associated with lipophilic carbon-containing compounds with molecular masses less than 300 Da and high equilibrium vapour pressures, known as volatile organic compounds (VOCs; Kanchiswamy et al., 2015). In addition to VOCs, microorganisms also release volatile inorganic compounds (VICs) with molecular masses less than 45 Da. When exogenously applied in discrete forms and low concentrations, some of these compounds can act as signalling molecules that promote plant growth, photosynthesis, and developmental changes through thiol redox modifications (Chen et al., 2011; He et al., 2008; Takahashi et al., 2014). Using a "box-in-box" cocultivation system in which plants are grown in the vicinity of microbial cultures covered with VOC-adsorbing charcoal filters, we have recently provided evidence that VOCs with molecular masses less than 40 Da and/or VICs other than CO_2 are strong determinants of plants' responses to microbial VCs (García-Gómez et al., 2019).

In *Arabidopsis* plants exposed to VCs emitted by various microbes, growth promotion is accompanied by increases in photosynthesis rates, levels of cytokinins (CKs) derived from the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, and starch in leaves, together with reductions in abscisic acid (ABA) contents, and distinct changes in shoot and root development (Ezquer et al., 2010; García-Gómez et al., 2019; Li et al., 2011; Sánchez-López, Baslam, et al., 2016 ; Zhang et al., 2008). Mutants with reduced CK and ABA sensitivity have been found to respond weakly to microbial VCs, indicating that plant responses to microbial VCs involve CK and ABA signalling (Sánchez-López, Baslam, et al., 2016; Zhang et al., 2008). Several studies have also found similarities in transcriptional changes in plants exposed to VCs emitted by different microorganisms, despite substantial differences in induced developmental changes in roots and shoots (García-Gómez et al., 2019; Sánchez-López, Baslam, et al., 2016). These findings suggest that regulation of some plant responses to microbial VCs is primarily posttranscriptional (García-Gómez et al., 2019). This hypothesis is supported by reports that fungal VCs do not affect the transcription of genes encoding most proteins that are differentially expressed following plants' exposure to VCs (Sánchez-López, Baslam, et al., 2016), and microbial VCs promote posttranslational reductive activation of starch biosynthetic enzymes (García-Gómez et al., 2019).

Reversible reduction–oxidation (redox) thiol modifications of cysteines (such as disulfide bond formation, *S*-glutathionylation, *S*-nitrosylation, and *S*-sulfenylation) provide fundamental posttranslational "switches" that play important roles in the regulatory mechanisms of metabolism, growth, and development that allow plants to adjust to continuously changing environmental constraints (Akter et al., 2015; Buchanan & Balmer, 2005; Couturier, Chibani, Jacquot, & Rouhier, 2013; Hu et al., 2015; Keech, Gardeström, Kleczkowski, & Rouhier, 2017). Therefore, identification of reactive cysteine residues is crucial not only for understanding protein functions but also for obtaining insights into the mechanisms involved in plants' responses to environmental changes. To assist such efforts, increasing numbers of mass spectrometry (MS)-based large-scale redox proteomic techniques have been developed recently to identify proteins that undergo reversible oxidative modifications (Akter et al., 2015; De Smet et al., 2019; Fares, Rossignol, & Peltier, 2011; Guo et al., 2014; Liu, Zhang, Wang, & Xia, 2014). The results obtained using these approaches indicate that almost any metabolic pathway may be subject to thiol-based redox regulation.

Important components of plants' thiol redox regulation machinery include thioredoxins (Trx) proteins that mediate disulfide–dithiol exchange of cysteine residues, thereby modulating activities of target proteins. Plant chloroplasts have a versatile set of Trxs, which receive reducing equivalents from the photosystem I (PSI) electron acceptor ferredoxin (Fdx), with participation of an Fdx-dependent Trx reductase (FTR; Schürmann & Buchanan, 2008). In addition, plastids contain an NADPH-dependent Trx reductase (NTR) with a joint Trx domain, called NTRC, which uses NADPH produced by the oxidative pentose phosphate pathway and photosynthetic electron transport (PET) as a source of reducing power (Serrato, Pérez-Ruiz, Spínola, & Cejudo, 2004). In *Arabidopsis*, NTRC is the most efficient reductant of two H_2O_2-detoxifying 2-Cys peroxiredoxins (Prxs) A and B, suggesting that it plays an important role in avoidance of toxic levels of reactive oxygen species (ROS; Kirchsteiger, Pulido, González, & Cejudo, 2009; Pérez-Ruiz et al., 2006; Puerto-Galán, Pérez-Ruiz, Guinea, & Cejudo, 2015; Pulido et al., 2010). Biochemical and genetic studies have provided evidence that NTRC participates in regulation of the redox status of stromal target proteins, including starch metabolism enzymes (Lepistö et al., 2013; Valerio et al., 2010), ATP synthase (Carrillo, Froehlich, Cruz, Savage, & Kramer, 2016; Nikkanen, Toivola, & Rintamäki, 2016), and diverse Trxs that control the redox status of Calvin–Benson cycle (CBC) enzymes such as plastidial fructose-1,6-bisphosphatase (cFBP1), phosphoribulokinase (PRK), sedoheptulose-1,7-bisphosphatase (SBP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Nikkanen et al., 2016; Ojeda et al., 2017; Yoshida & Hisabori, 2016). NTRC also regulates the redox status of Mg–protoporfirin IX methyltransferase (CHLM), which is involved in chlorophyll biosynthesis (Da et al.,

2017; Richter et al., 2013), and CHLI (Pérez-Ruiz, Guinea, Puerto-Galán, & Cejudo, 2014), which is the subunit of the Mg–chelatase complex involved in chlorophyll biosynthesis and ABA signalling (Du et al., 2012; Tsuzuki et al., 2011). Furthermore, recent studies have shown that NTRC plays important roles in control of nonphotochemical quenching (NPQ) and PET (Carrillo et al., 2016; Naranjo et al., 2016).

Arabidopsis NTRC knockout (*ntrc*) mutants show a strong photoperiod-dependent phenotype of stunted growth and low chlorophyll content, highlighting the enzyme's crucial roles in chloroplast functions, photosynthesis, and growth (Lepistö, Kangasja, & Luomala, 2009; Pérez-Ruiz et al., 2006). NTRC is localized in plastids of both photosynthetic and nonphotosynthetic tissues, prompting the hypothesis that it acts as a general molecular switch capable of converting reducing power in the form of NADPH into redox signals that coordinate redox regulation in the whole plant (Kirchsteiger, Ferrández, Pascual, González, & Cejudo, 2012). Growth is dramatically inhibited in *Arabidopsis* mutants combining deficiencies of NTRC and Trxs *f* and *x* (Ojeda et al., 2017), suggesting that NTRC and the Fdx–FTR–Trxs systems act in concert. Recent studies have shown that the *ntrc* phenotype is suppressed by decreased expression of 2-Cys Prxs (Ojeda, Pérez-Ruiz, & Cejudo, 2018; Pérez-Ruiz, Naranjo, Ojeda, Guinea, & Cejudo, 2017). These findings led to the proposal that 2-Cys Prxs are crucial to maintain the redox status of the pool of plastidial Trxs and, consequently, the light-dependent reduction of photosynthesis-related proteins (Ojeda et al., 2018; Pérez-Ruiz et al., 2017). Accordingly, it has been suggested that the phenotype of *ntrc* plants is due to impairment of the redox regulation of chloroplastic growth- and photosynthesis-related processes rather than ROS over-accumulation as a consequence of 2-Cys Prxs inactivation (Pérez-Ruiz et al., 2017).

Microbial VCs induce smaller increases in starch contents in *ntrc* plants than in wild-type (WT) plants (Li et al., 2011). Because NTRC plays a central role in redox regulation of various plastidial processes involved in metabolism, growth, and development, we hypothesized that NTRC could be involved in plants' overall responses to VCs. To test the potential importance of posttranslational thiol modifications of proteins in plant responses to microbial VCs and investigate NTRC's contribution to these responses, we compared developmental, metabolic, hormonal, and redox proteomic responses of WT and *ntrc* plants to VCs emitted by the fungal phytophathogen *Alternaria alternata*. Our findings show that NTRC is an important mediator of plant responses to fungal VCs and provide a broad quantitative picture of redox-mediated responses of the thiol proteome in *Arabidopsis* to VCs, allowing us to infer potential redox switches.

2 | MATERIALS AND METHODS

2.1 | Plant and microbial cultures, growth conditions, and sampling

The work was carried out using *Arabidopsis thaliana* L. (Heynh) ecotype Columbia (Col-0), the *ntrc* mutant (Pulido et al., 2010), the *Δ2cp* and *ntrc–Δ2cp* mutants with severe reduction of 2-Cys Prxs expression (Pérez-Ruiz et al., 2017), the ADPglucose pyrophosphorylase null *aps1* mutant (SALK_040155), and the *gwd* (SALK_077211) mutant. Microorganisms used in this study were also used by Sánchez-López, Baslam, et al. (2016). Unless otherwise indicated, the plants were cultured in Petri dishes (92 × 16 mm, Sarstedt, Ref. 82.1472.001) containing sucrose-free half-strength solid Murashige and Skoog (Phytotechlab M519) medium in growth chambers providing "long-day" 16-hr light (90 μmol photons s^{-1} m^{-2}), 22°C/8-hr dark, 18°C cycles. Fungi were cultured in small Petri dishes (35 × 10 mm, Sarstedt, Ref. 82.1135.500) containing solid Murashige and Skoog medium supplemented with 90-mM sucrose. Bacteria were cultured in small Petri dishes containing solid M9 minimal (95-mM Na_2HPO_4/44-mM KH_2PO_4/17-mM NaCl/37-mM NH_4Cl/0.1-mM $CaCl_2$/2-mM $MgSO_4$ and 1.5% bacteriological agar) medium supplemented with 50-mM glucose. M9 medium for *Bacillus subtilis* culture was supplemented with 7 μM each of $MnSO_4$, $FeSO_4$, and $ZnSO_4$ and 1 μM of thiamine. Effects of microbial VCs on plants were investigated using the "box-in-box" cocultivation system as described in García-Gómez et al. (2019). Briefly, microbial cultures in unlidded Petri dishes with a top layer of VOC-adsorbing activated charcoal filters and plant cultures of 14 days after sowing were placed together in sterile plastic boxes (200 × 150 × 50-mm IT200N Instrument Trays; AW Gregory, UK) sealed with polyvinyl chloride plastic wrap. As negative controls, Petri dishes containing plant cultures were incubated in sealed boxes together with Petri dishes containing sterile microbial culture media and a charcoal filter. At the indicated incubation periods, leaves were harvested, immediately freeze clamped, and ground to a fine powder in liquid nitrogen with a pestle and mortar.

2.2 | Determination of gas exchange rates and photosynthetic parameters

Gas exchange rates were determined as described by Sánchez-López, Baslam, et al. (2016) using a LI-COR 6400 gas exchange portable photosynthesis system (LI-COR, Lincoln, NE, USA). The net rate of CO_2 assimilation (A_n) was calculated using equations developed by von Caemmerer and Farquhar (1981). The maximum rate of carboxylation by Rubisco (V_{cmax}), triose phosphate use (*TPU*), and maximum electron transport demand for RuBP regeneration (J_{max}) values were calculated from A_n/C_i curves (where C_i is the intracellular CO_2 concentration) according to Long and Bernacchi (2003). Photosynthetic electron transport (ETR) values were calculated according to Krall and Edwards (1992) as Φ_{PSII} × PPFD × 0.84 × 0.5, where PPFD is the photosynthetic photon flux density incident on the leaf, Φ_{PSII} is the photosystem II (PSII) operating efficiency, 0.5 was used as the fraction of excitation energy distributed to PSII (Ögren & Evans, 1993), and 0.84 was used as the fractional light absorbance (Morales, Abadia, & Abadia, 1991). Chlorophyll fluorescence emission parameters were determined using a PlantScreen™ XYZ System (Photon Systems Instruments, Brno, Czech Republic). The phenotyping system was equipped with a FluorCam unit for pulse amplitude modulated measurement of chlorophyll fluorescence. After 20 min of dark adaptation,

the standardized measurement protocol was applied, as described by Humplík et al. (2015). The maximum quantum yields of PSII in the dark-adapted state (Φ_{Po}; also referred as F_v/F_m), Φ_{PSII}, and nonphotochemical quenching (Φ_{NPQ}) were calculated from the measured parameters according to Lazár (2015).

2.3 | Root morphological analysis

Numbers and lengths of roots of plants grown on vertical square Petri dishes (10 × 10 × 2 cm, Sarstedt, Ref. 82.9923.422) were measured using a MVX10 stereomicroscope, and microphotographs were captured with a DP72 video camera equipped with Cell D software (all supplied by Olympus, Japan).

2.4 | Analytical procedures

For sucrose, glucose, and fructose measurements, a 0.1-g aliquot of the frozen powders (see above) were resuspended in 1 ml of 90% ethanol, incubated at 70°C for 90 min, and centrifuged at 13,000 × *g* for 10 min. Sugar contents in the supernatants were then determined by high-performance liquid chromatography with pulsed amperometric detection using an ICS-3000 Dionex system as described by Bahaji et al. (2015). NADP and NADPH contents were measured as described by Queval and Noctor (2007). Recovery percentages of the analytes were estimated from differences in measured amounts in samples of frozen tissue slurry with and without spiking by standards immediately after addition of extraction solutions. All presented concentrations of these metabolites were corrected for losses during extraction. Starch was measured using an amyloglucosydase-based test kit (Boehringer Mannheim, Germany), and chlorophyll contents were quantified according to Lichtenthaler (1987). To determine levels of CKs, portions of the frozen leaves (prepared as described above) were lyophilized, and CKs were quantified following Novák, Hauserová, Amakorová, Dolezal, and Strnad (2008). ABA contents were determined as described by Floková et al. (2014).

2.5 | ROS staining

ROS were semiquantitatively detected in rosettes essentially as described by Nguyen et al. (2017). Briefly, superoxide anion was detected by staining rosettes for 15 min with 0.05% nitro blue tetrazolium (NBT; w/v) in 50-mM potassium phosphate, pH 7.0, and H_2O_2 by staining for 5 hr with 0.1% 3,3′-diaminobenzidine (DAB) in 10-mM potassium phosphate, pH 7.0.

2.6 | Real-time quantitative PCR

Total RNA was extracted from frozen *Arabidopsis* leaves of in vitro cultured plants using the TRIzol method according to the manufacturer's recommendations (Invitrogen), following treatment with RNAase-free DNAase (Takara). RNA (1.5 μg) was reverse transcribed using polyT primers and an Expand Reverse Transcriptase kit (Roche) according to the manufacturer's instructions. RT-PCR amplification was performed as described by Sánchez-López, Baslam, et al. (2016) using primers listed in Table S1, and their specificity was checked by separating the obtained products on 1.8% agarose gels.

2.7 | Identification of redox-sensitive cysteines

2.7.1 | Protein extraction and tagging

Proteins were extracted and tagged essentially as described by Guo et al. (2014) with some modifications. Briefly, 0.5 g of plant tissue was ground in liquid nitrogen, mixed with 2-ml extraction buffer (20-mM HEPES, 0.5-mM EDTA, 0.5% Triton X-100, 100-mM NaCl, and 1% SDS) supplemented with 100-mM *N*-ethylmaleimide (NEM) to block the free thiol groups, and incubated for 1 hr at room temperature. The mixture was centrifuged at 4°C for 10 min at 10,000 × *g*. Proteins were precipitated and washed to remove any free NEM by adding of 5 volumes of iced acetone and incubating overnight at −20°C. After centrifugation, the supernatant was discarded, and the protein pellet was recovered by centrifugation at 10,000 × *g* and 4°C for 10 min, rinsed with cold acetone three times, and air dried. Urea solution (8 M) was added to dissolve the pellet, and brief intermittent sonication was applied until the pellet dissolved. The resulting protein-containing solution was incubated with 10-mM dithiothreitol (DTT) at 37°C for 30 min to reduce oxidized thiols. DTT was then removed with PD-10 desalting columns (GE Healthcare). The protein concentration of each preparation was determined using a Pierce™ 660 nm Protein Assay Kit (Thermo Fisher Scientific), and a portion containing 500 μg of proteins was used for the subsequent enrichment experiment. The samples were alkylated with 1 M of 2-iodoacetamide (IAM; Wako) for 1 hr at 37°C in the dark to tag the DTT-reduced thiols by carbamidomethylation. Proteins were acetone precipitated, resolubilized in 8-M urea, digested with lysine (Sigma) for 3 hr and trypsin (Sigma) overnight at 37°C at a 1:40 trypsin-to-protein mass ratio, desalted using Sep-Pak C18 cartridges, and dried in a SpeedVac.

2.7.2 | iTRAQ labelling of peptides and strong cation exchange fractionation

Peptides from samples of non-VC-treated WT plants, VC-treated WT plants, non-VC-treated *ntrc* plants, and VC-treated *ntrc* plants were labelled at room temperature for 2 hr with iTRAQ reporter reagents 114, 115, 116, and 117, respectively, according to the iTRAQ manual (SCIEX). All four samples were then mixed in a single tube and dried in a SpeedVac (Eppendorf). Peptides were subsequently fractionated by strong cation exchange columns (SCIEX, USA) and then lyophilized to dryness.

2.7.3 | Liquid chromatography–mass spectrometry analysis

Proteomic profiles of three technical replicates of samples were quantitatively analysed as described by Fukao et al. (2011) and Shiraya

et al. (2015) using a coupled DiNA-A (KYA Tech., Tokyo, Japan) and LTQ-Orbitrap XL (Thermo Fisher Scientific) liquid chromatography–MS/MS system. The ionization voltage and capillary transfer temperature at the electrospray ionization nanostage were set to 1.7–2.5 kV and 200°C, respectively. iTRAQ-labelled peptides were separated in a MonoCap C18 High Resolution 2000 column (GL Sciences), using a mobile phase consisting of a 240-min linear gradient of 2–26.7% acetonitrile in water (with a 10-min wash with 80% acetonitrile, v/v, and 15-min re-equilibration between each run, maintaining 0.1% formic acid, v/v, throughout). Peptides that eluted from the column were introduced directly into the mass at a flow rate of 300 nl min^{-1}. Divalent or trivalent ions were subjected to MS/MS analysis in top 3 data-dependent acquisition mode. Collision-induced dissociation and higher energy collisional dissociation settings for all MS/MS analyses were 35 and 45 eV, respectively.

2.7.4 | MS data analysis

Raw MS data files were submitted to Proteome Discoverer 1.4 with the SEQUEST algorithm (Thermo Scientific Inc., Bremen, Germany) and MS Amanda (Dorfer et al., 2014) to search for matches in the Gene Ontology *Arabidopsis* database. Percolator was set to a target false discovery rate of 0.05. Peptides were quantified using the iTRAQ4plex reporter ion method and identified from database search results with the following criteria: enzyme, trypsin; maximum missed cleavage sites, 2; peptide charge, 2+ or 3+; MS tolerance, 5 ppm; MS/MS tolerance, ±0.6 Da; and dynamic modification, oxidation (H, M, and W) and iTRAQ 4-plex (K, Y, and N-terminus). Variable modifications for the search included carbamidomethylation and NEM at cysteines, iTRAQ (4-plex) at tyrosines, and oxidation at methionines (mass shifts: 57.02, 125.05, 144.10, and 15.99, respectively). The fixed modification was iTRAQ (4-plex) reagent labelling at N-terminal and lysine.

2.7.5 | OxiTRAQ data analysis

Amounts of detected peptides were normalized using total intensities of their assigned mass spectra according to the SEQUEST searching results, and peptide ratios were calculated accordingly from medians of the normalized intensities. Log_2-transformed treated/control ratios were subjected to Student's *t* test (two tailed). Differences in concentrations of peptides between samples with fold changes greater than 1.9 or less than 0.5, and $P < 0.05$, were considered significantly changed. The isobaric tags for relative and absolute quantitation (iTRAQ) data (Parker, Zhu, Zhu, & Chen, 2012) were searched using the database mentioned above to generate information about changes in total protein levels. The same set of samples were analysed in another MS run and used as technical replicates to determine possible variation caused by MS analysis. Student's *t* test was applied to peak intensity values of all spectra of corresponding peptides from control and treated samples in the same MS run.

2.8 | Statistical analysis

Presented data are means (± standard error) obtained from three to four independent experiments, with 3–5 replicates for each experiment. The significance of differences between WT and *ntrc* plants not exposed to VCs and plants exposed and not exposed to *A. alternata* VCs was statistically evaluated with Student's *t* test using the SPSS software. Differences were considered significant if $P < .05$.

3 | RESULTS

3.1 | *ntrc* plants poorly respond to *A. alternata* VCs

Using the box-in-box cocultivation system described by García-Gómez et al. (2019), we compared growth and developmental responses of WT and *ntrc* plants grown in the vicinity of *A. alternata* cultures covered with VOC-adsorbing charcoal filters. We also analysed the growth response to fungal VCs of near starch-less *aps1* plants impaired in starch biosynthesis and starch-excess *gwd* plants impaired in starch breakdown (Baslam et al., 2017; Caspar et al., 1991; Ventriglia et al., 2008). In the absence of VOC-depleted fungal VCs, rosettes and roots of the *ntrc* mutant were slightly smaller and lighter than those of WT plants (Figure 1a,b), as reported by Lepistö et al. (2009) and Li et al. (2011). *ntrc* plants produced shorter primary roots and fewer first- and second-order lateral roots (LRs) than WT plants (Figure 1a,c), as found by Kirchsteiger et al. (2012). In keeping with findings by Sánchez-López, Baslam, et al. (2016) and García-Gómez et al. (2019), VOC-depleted fungal VCs promoted rosette and root growth, elongation of the primary root, and formation of first- and second-order LRs in WT plants (Figure 1), thereby increasing the density of the root system (Figure 1c). As in WT plants, VOC-depleted fungal VCs promoted rosette and root growth in *aps1* and *gwd* plants (Figure S1). Notably, fungal VC-promoted rosette and root growth was substantially weaker in *ntrc* plants than in WT plants (Figure 1a,b). Furthermore, unlike in WT plants, VOC-depleted fungal VCs did not promote primary root elongation or LR formation and did not enhance the root system density in the *ntrc* mutant (Figure 1c). Fungal VCs promoted root hair elongation in both WT and *ntrc* plants (Figure S2).

It is known that VCs emitted by diverse types of microorganisms promote growth of WT plants (Sánchez-López, Baslam, et al., 2016). To assess further the growth responses of *ntrc* plants to microbial VCs, we grew plants in the presence of adjacent cultures of phylogenetically diverse species of fungi and bacteria with or without a top layer of VOC-adsorbing charcoal filter. As shown in Figure S3, growth promoted by VOC-depleted VCs of tested microorganisms was substantially weaker in *ntrc* plants than in WT plants. In keeping with García-Gómez et al. (2019), the responses of WT and *ntrc* plants grown in the vicinity of microbial cultures not covered with charcoal filters were identical to those of plants grown with adjacent fungal cultures covered with charcoal filters (not shown).

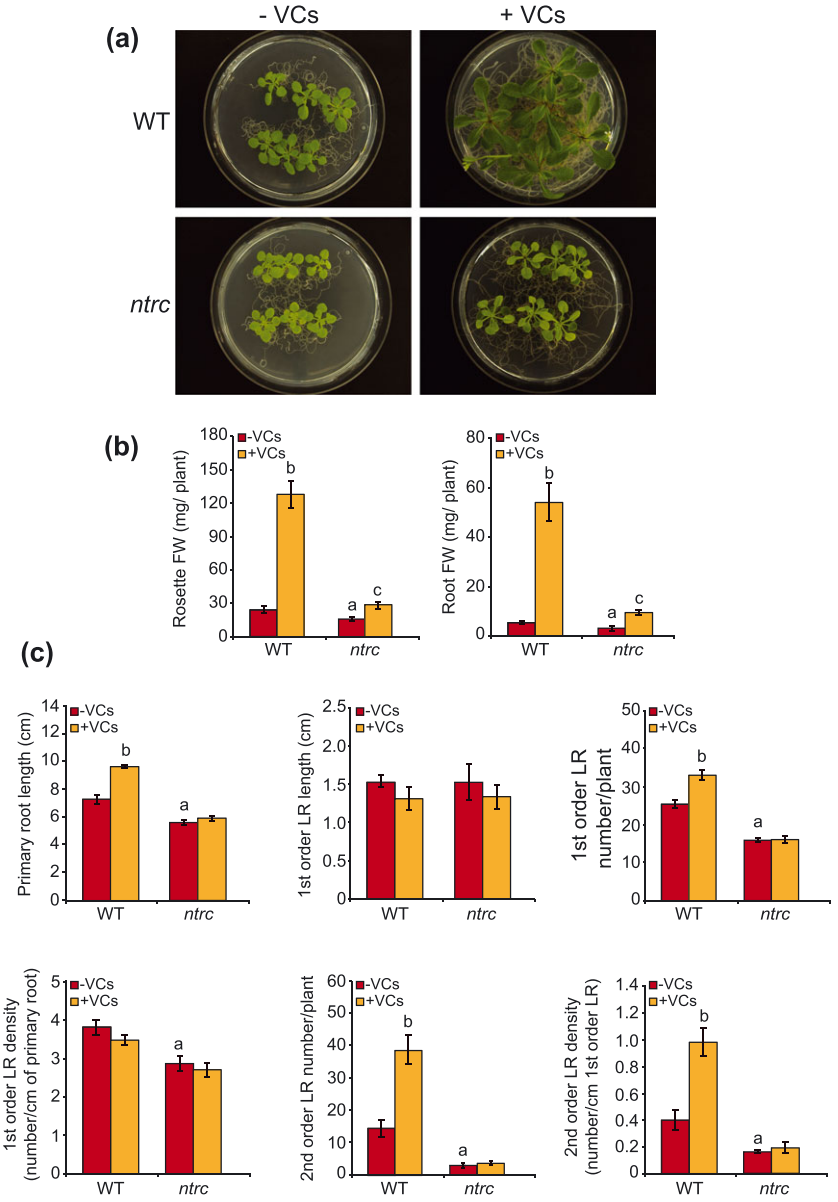

FIGURE 1 *ntrc* plants weakly respond to *Alternaria alternata* volatile compounds (VCs). (a) external phenotypes, (b) rosette and root fresh weight (FW), and (c) root architecture parameters of wild-type (WT) and *ntrc* plants cultured in the absence or continuous presence of VCs emitted by adjacent *A. alternata* cultures covered with volatile organic compound-adsorbing charcoal filters for 1 week. Values in (b) and (c) are means ± standard error for three biological replicates (each a pool of 12 plants) obtained from four independent experiments. [a]Significant differences, according to Student's *t* test ($P < .05$), between WT and *ntrc* plants cultured without fungal VC treatment. [b]Significant differences, according to Student's *t* test ($P < .05$), between VC-treated and nontreated WT plants. [c]Significant differences, according to Student's *t* test ($P < .05$), between VC-treated and nontreated *ntrc* plants

3.2 | *A. alternata* VCs reduce ABA content and increase levels of plastidial CKs but weakly alter expression of ABA- and CK-responsive genes in *ntrc* plants

Having established NTRC's involvement in plants' response to fungal VCs, we compared their effects on ABA and CK contents and signalling in *ntrc* and WT plants. For this, we measured the contents of ABA and CKs in mature leaves of *ntrc* plants cultured in the absence or continuous presence of VCs emitted by adjacent *A. alternata* cultures for 3 days. We also analysed expression levels of a selected group of CK- and ABA-responsive genes (Bhargava et al., 2013; Brenner & Schmülling, 2015; Nemhauser, Hong, & Chory, 2006) that are sensitive to fungal VCs (cf. table 3 in Sánchez-López, Baslam, et al., 2016) by qRT-PCR.

In the absence of fungal VCs, *ntrc* leaves had higher ABA content than WT leaves (209 ± 16 and 352 ± 8 pmol g^{-1} dry weight in WT and *ntrc* plants, respectively) but similar CK contents (Tables 1 and S2 and Figure S4). *A. alternata* VCs caused a significant reduction in ABA contents in leaves of both WT and *ntrc* plants (86.1 ± 19.7 and 94.7 ± 25.4 pmol g^{-1} dry weight, respectively) and increases in levels of precursors and active and transport forms of plastidial CKs (Tables 1 and S2 and Figure S4). Notably, changes in levels of expression of ABA- and CK-responsive genes in response to VC treatment were substantially weaker in *ntrc* leaves than in WT leaves (Figure 2).

3.3 | Fungal VCs weakly increase photosynthetic capacities of exposed *ntrc* plants

NTRC plays an important role in photosynthesis (Naranjo et al., 2016; Nikkanen et al., 2016; Ojeda et al., 2017). To investigate whether *ntrc* plants' weak responses to VCs could be due to nonresponsiveness of photosynthesis to fungal VCs, we compared key photosynthetic

TABLE 1 Contents of precursors and active and transport forms of plastidial CK (pmol g^{-1} dry weight) in leaves of WT and *ntrc* plants cultured in solid Murashige and Skoog medium in the absence or continuous presence of VCs emitted by adjacent *A. alternata* cultures for 3 days

	MEP pathway (plastid)-derived CKs			
CKs	**WT − VCs**	**WT + VCs**	***ntrc* − VCs**	***ntrc* + VCs**
iPRMP	51.5 ± 1.0	132 ± 6[b]	49.8 ± 1.9	76.6 ± 2.3[c]
tZRMP	5.66 ± 0.43	53.0 ± 2.8[b]	2.93 ± 0.12[a]	8.75 ± 0.48[c]
iPR	8.32 ± 0.18	13.3 ± 16.7	14.5 ± 1.7[a]	68.7 ± 7.4[c]
tZR	1.60 ± 0.08	13.0 ± 12.0[b]	1.45 ± 0.04	6.89 ± 0.54[c]
DZR	2.47 ± 0.13	0.65 ± 0.09	2.95 ± 0.32	3.96 ± 0.50
iP	16.2 ± 0.5	15.1 ± 1.3	19.3 ± 2.8	21.7 ± 2.4
tZ	13.7 ± 1.8	24.0 ± 1.3[b]	16.0 ± 2.7	21.7 ± 3.6
DZ	0.71 ± 0.15	0.65 ± 0.09	0.89 ± 0.20	0.77 ± 0.10

Note. Values are means ± standard error of determinations in three independent experiments.

Abbreviations: CK, cytokinin; DZ, dihydrozeatin; DZR, dihydrozeatin riboside; iP, isopentenyladenine; iPR, N^6-isopentenyladenosine; iPRMP, N^6-isopentenyladenosine-5′-monophosphate; tZ, *trans*-zeatin; tZR, *trans*-zeatin riboside; tZRMP, *trans*-zeatin riboside 5′-monophosphate; VCs, volatile compounds; WT, wild-type.

[a]Significant differences, according to Student's *t* test ($P < .05$), between WT and *ntrc* plants cultured without fungal VC treatment.

[b]Significant differences, according to Student's *t* test ($P < .05$), between VC-treated and nontreated WT plants.

[c]Significant differences, according to Student's *t* test ($P < .05$), between VC-treated and nontreated *ntrc* plants.

parameters of WT and *ntrc* plants cultured in the absence or continuous presence of VCs emitted by adjacent *A. alternata* cultures.

As shown in Figure 3a, and previously recorded (Pérez-Ruiz et al., 2006), leaves of *ntrc* plants that were not exposed to fungal VCs accumulated lower levels of chlorophyll than those of WT plants. In keeping with previous reports (Lepistö et al., 2009; Naranjo et al., 2016), Φ_{Po}, Φ_{PSII}, and photochemical quenching (*qP*) were lower in *ntrc* plants than in WT plants (Table 2 and Figure S5). Accordingly, A_n and ETR at all C_i levels (Figure 3b, c) as well as V_{cmax}, *TPU*, and J_{max} values (Table 2) were lower in *ntrc* plants than in WT plants. Nonphotochemical quenching of chlorophyll fluorescence (Φ_{NPQ}) was higher in *ntrc* plants than in WT plants (Table 2), as previously reported by Naranjo et al. (2016). The NADPH/NADP ratio was higher in *ntrc* leaves than in WT leaves (Figure 3d), as observed by Thormählen et al. (2015). Leaves of VC-exposed WT plants accumulated substantially higher levels of chlorophyll than those of nontreated plants (Figure 3a). Φ_{PSII} was higher and Φ_{NPQ} lower in VC-treated WT plants than in nontreated controls (Table 2), as found by Sánchez-López, Baslam, et al. (2016). Accordingly, leaves of VC-treated WT plants had higher A_n and ETR values than controls at all C_i levels (Figure 3b,c). VC-treated WT leaves also had higher V_{cmax}, J_{max}, and *TPU* values than nontreated controls (Table 2), higher NADPH/NADP ratios (Figures 3d and S6), and higher levels of primary photosynthates (e.g., glucose, fructose, and sucrose; Figure 3e). In contrast, *A. alternata* VCs caused a nonsignificant reduction in chlorophyll contents in leaves of *ntrc* plants (Figure 3a). Moreover, VCs had a negative effect on φ_{Po} and *qP* and did not significantly alter Φ_{NPQ} in *ntrc* plants (Table 2). Furthermore, no significant differences were observed in A_n values between VC-treated and nontreated *ntrc* leaves at any C_i levels (Figure 3b). Accordingly, no significant differences in V_{cmax}, J_{max}, and *TPU* were observed between VC-treated and nontreated *ntrc* leaves (Table 2). Finally, NADPH/NADP ratios were lower in leaves of VC-treated *ntrc* plants than in controls (Figures 3d and S6), and levels of primary photosynthates were not significantly altered by *A. alternata* VCs in the *ntrc* mutant (Figure 3e). Fungal VCs enhanced ETR at all C_i levels and Φ_{PSII} in leaves of *ntrc* plants (Table 2, Figure 3c).

3.4 | The weak response to fungal VCs of *ntrc* plants is suppressed by decreased 2-Cys Prx expression

Pérez-Ruiz et al. (2017) have recently reported that reduction of 2-Cys Prx expression suppresses the *ntrc* phenotype and proposed that NTRC regulates chloroplast redox status via its functional relationship with 2-Cys Prxs. To investigate the possible involvement of the

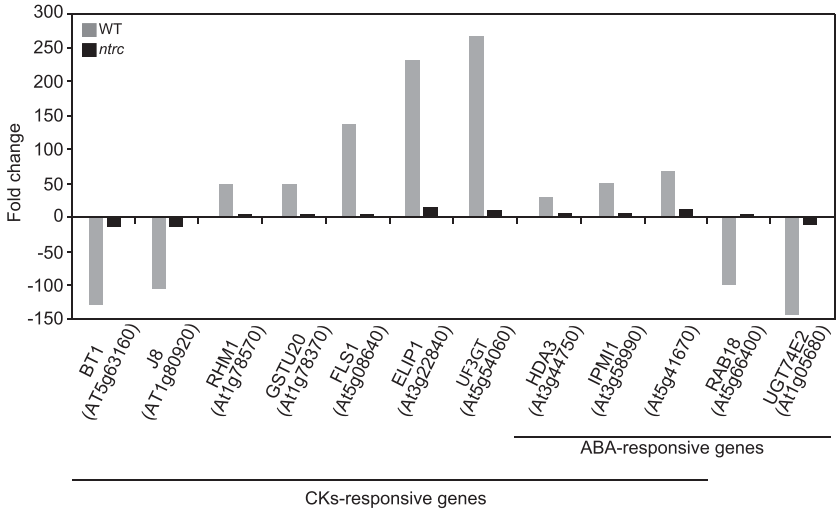

FIGURE 2 *Alternaria alternata* volatile compounds (VCs) weakly alter expression of abscisic acid (ABA)- and cytokinin (CK)-responsive genes in *ntrc* plants. Relative abundance of transcripts of CK- and ABA-responsive genes in leaves of WT and *ntrc* plants (grey and black, respectively) cultured in the absence or continuous presence of VCs emitted by adjacent *A. alternata* cultures for 3 days. Fold change values are differences in levels of transcripts (measured by quantitative RT-PCR) in leaves of plants cultured in the presence of VCs and harvested at the end of the light period for 16 hr, relative to those of control leaves of plants cultured in the absence of VCs. WT, wild-type

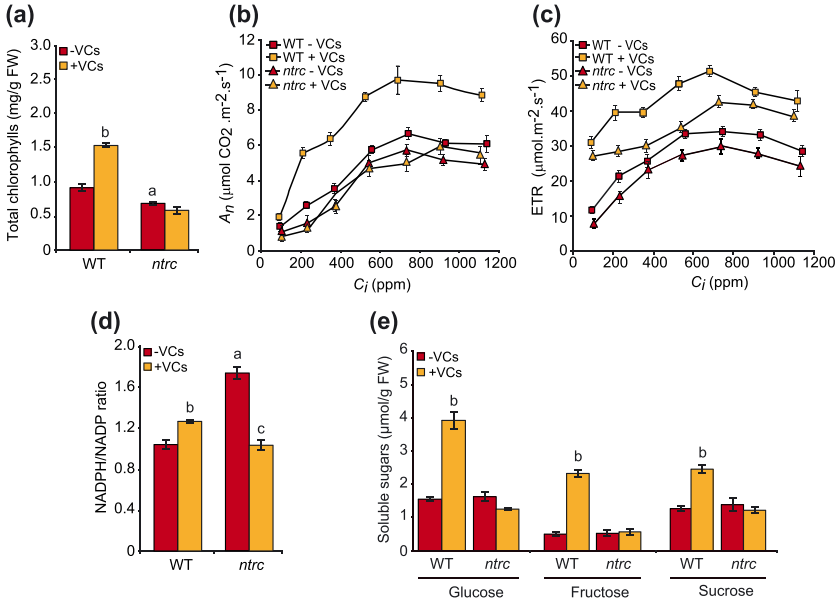

FIGURE 3 Fungal volatile compounds (VCs) poorly increase photosynthetic capacities of exposed *ntrc* plants. (a) Total chlorophyll contents, curves of (b) net CO_2 assimilation rate (A_n) and (c) photosynthetic electron transport (ETR) versus intercellular CO_2 concentration (C_i), (d) NADPH/NADP ratio, and (e) levels of primary photosynthates (soluble sugars) in leaves of wild-type (WT) and *ntrc* plants cultured in the absence or continuous presence of VCs emitted by adjacent *Alternaria alternata* cultures for 1 week. VC treatment started 28 days after sowing plants. Values in (a), (d), and (e) are means ± standard error for three biological replicates (each a pool of 12 plants) obtained from four independent experiments. [a]Significant differences, according to Student's *t* test ($P < .05$), between WT and *ntrc* plants cultured without fungal VC treatment. [b]Significant differences, according to Student's *t* test ($P < .05$), between VC-treated and nontreated WT plants. [c]Significant differences, according to Student's *t* test ($P < .05$), between VC-treated and nontreated *ntrc* plants. FW, fresh weight

TABLE 2 Photosynthetic parameters of leaves of WT and *ntrc* plants cultured in the absence or continuous presence of VCs emitted by adjacent *A. alternata* cultures for 3 days

Treatment	φ_{Po}	φ_{PSII}	φ_{NPQ}	*qP*	J_{max} (µmol e^- m^{-2} s^{-1})	V_{cmax} (µmol CO_2 m^{-2} s^{-1})	*TPU* (µmol Pi m^{-2} s^{-1})
WT − VCs	0.830 ± 0.002	0.710 ± 0.001	0.243 ± 0.002	0.722 ± 0.008	33.1 ± 1.7	12.8 ± 0.9	1.82 ± 0.09
WT + VCs	0.831 ± 0.000	0.718 ± 0.002[b]	0.215 ± 0.002[b]	0.770 ± 0.002[b]	53.7 ± 1.3[b]	20.4 ± 2.0[b]	2.79 ± 0.04[b]
ntrc − VCs	0.815 ± 0.001[a]	0.688 ± 0.003[a]	0.311 ± 0.004[a]	0.543 ± 0.010[a]	28.6 ± 2.1[a]	11.3 ± 0.8[a]	1.44 ± 0.05[a]
ntrc + VCs	0.795 ± 0.002[c]	0.696 ± 0.004	0.304 ± 0.009	0.362 ± 0.013[c]	29.0 ± 2.3	11.6 ± 0.7	1.36 ± 0.02

Note. Values are means ± standard error of determinations in three independent experiments.

Abbreviations: VCs, volatile compounds; WT, wild-type.

[a]Significant differences, according to Student's *t* test ($P < .05$), between WT and *ntrc* plants cultured without fungal VC treatment.

[b]Significant differences, according to Student's *t* test ($P < .05$), between VC-treated and nontreated WT plants.

[c]Significant differences, according to Student's *t* test ($P < .05$), between VC-treated and nontreated *ntrc* plants.

NTRC–2-Cys–Prx tandem in the plant response to fungal VCs, we characterized *Δ2cp* and *ntrc–Δ2cp* plants cultured in the absence or continuous presence of charcoal-filtered VCs emitted by adjacent *A. alternata* cultures for 1 week. As shown in Figure 4a, VC-exposed *Δ2cp*, *ntrc–Δ2cp*, and WT plants had similar sizes and were much larger than the nontreated controls. Leaves of VC-exposed *Δ2cp* and *ntrc–Δ2cp* plants accumulated higher levels of chlorophyll, had higher A_n values, and accumulated higher levels of primary photosynthates (starch and soluble sugars) than leaves of nontreated plants (Figure 4 b–d). These findings clearly indicate that the suppressor effect of the *ntrc* phenotype by decreased levels of 2-Cys Prxs is also exerted on the plant's response to VOC-depleted fungal VCs. To test whether the weak responsiveness to VOC-depleted fungal VCs of *ntrc* plants could be due to ROS over-accumulation in leaves, we examined their superoxide anion and H_2O_2 contents by NBT and DAB staining, respectively. No significant differences in ROS contents were found between leaves of WT, *ntrc*, *Δ2cp*, and *ntrc–Δ2cp* plants and VC-treated and -untreated plants (Figure S7).

3.5 | *A. alternata* VCs promote global reduction and oxidation of the thiol redox proteomes of WT and *ntrc* leaves, respectively

The weak response to VCs of the *ntrc* mutant suggested that thiol-dependent posttranslational modifications play an important role in

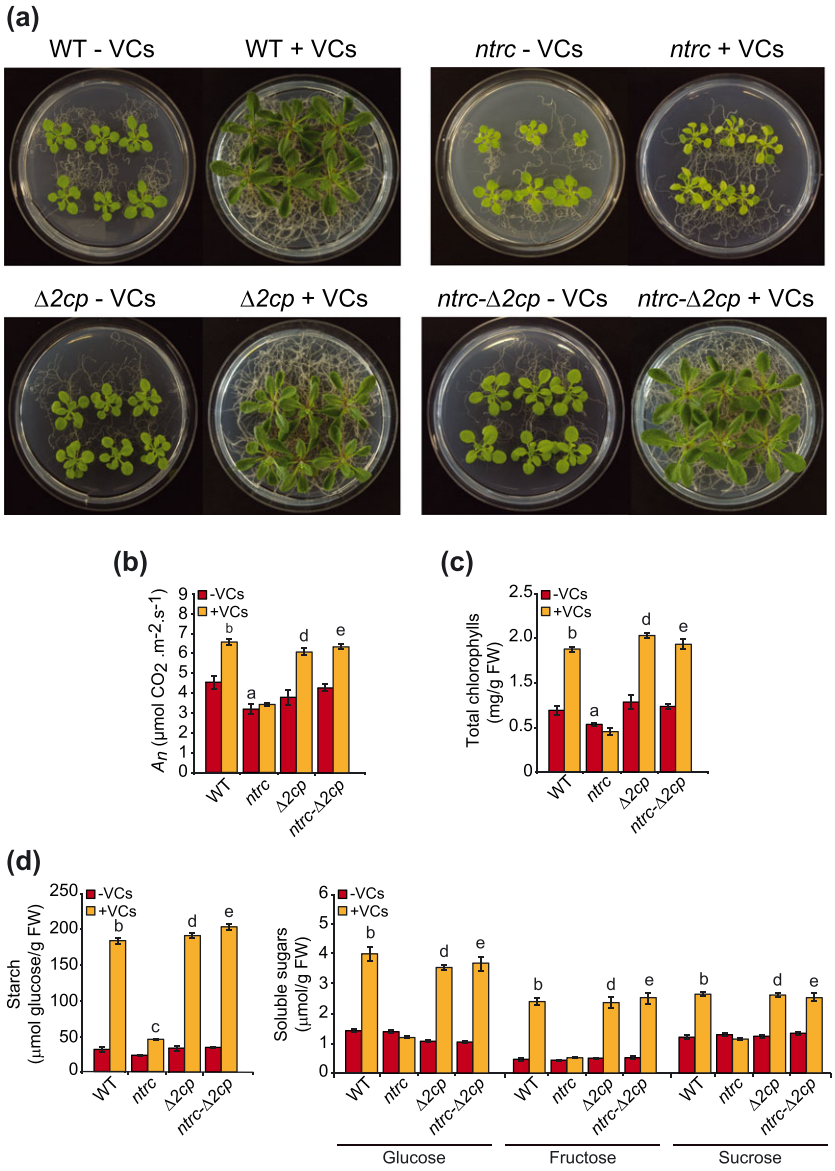

FIGURE 4 Reducing 2-Cys Prx activity restores *ntrc* plants' responses to fungal volatile compounds (VCs). (a) external phenotype, (b) net CO_2 assimilation rate (*An*), (c) total chlorophyll content, and (d) contents of primary photosynthates (starch, sucrose, glucose, and fructose) in leaves of wild-type (WT), *ntrc*, *Δ2cp*, and *ntrc–Δ2cp* plants cultured in the absence or continuous presence of VCs emitted by adjacent *Alternaria alternata* cultures for 1 week. Values in panels (b), (c), and (d) are means ± standard error of three biological replicates (each a pool of 12 plants) obtained from four independent experiments. [a]Significant differences, according to Student's *t* test ($P < .05$), between WT and *ntrc*, *Δ2cp*, and *ntrc–Δ2cp* plants cultured without fungal VC treatment. [b]Significant differences, according to Student's *t* test ($P < .05$), between VC-treated and nontreated WT plants. [c]Significant differences, according to Student's *t* test ($P < .05$), between VC-treated and nontreated *ntrc* plants. [d]Significant differences, according to Student's *t* test ($P < .05$), between VC-treated and nontreated *Δ2cp* plants. [e]Significant differences, according to Student's *t* test ($P < .05$), between VC-treated and nontreated *ntrc–Δ2cp* plants. FW, fresh weight

plant responses to VCs. Thus, we next addressed the possibility that the different responses to *A. alternata* VCs observed in WT and *ntrc* plants include (and are partially due to) differences in oxidative modifications of protein cysteine residues. Because of the complexity of the multiple forms of cysteine redox modifications (e.g., disulfide, *S*-nitrosylation, *S*-glutathionylation, *S*-cyanylation, persulfidation, or *S*-sulfenylation), this study focused on the quantification of total reversible thiol oxidation in leaves of WT and *ntrc* plants cultured in the absence or continuous presence of VCs emitted by nearby fungal cultures for 3 days using OxiTRAQ-based quantitative redox proteomics. This approach is based on differential labelling of reduced and oxidized cysteines and only quantifies reversibly oxidized thiols that are DTT reducible (for further details, see Section 2).

In total, 782 unique cysteine-containing peptides from 747 distinct proteins obtained from WT leaves and 781 unique cysteine-containing peptides from 746 distinct proteins obtained from *ntrc* leaves were identified in this study (Tables S3 and S4). A peptide, whose abundance differed by more than 1.9-fold or less than 0.5 with a $P < .05$, was considered to be redox-sensitive in this report. Applying this criterion, 113 cysteine-containing peptides from 110 distinct proteins and 87 cysteine-containing peptides from 87 distinct proteins were found to be redox sensitive in response to VCs in WT and *ntrc* plants, respectively (Tables S5 and S6). Of the VC-responsive, redox-sensitive proteins, nearly 15% have been shown to be redox sensitive in other studies (Tables S5 and S6). Among the 113 VC-responsive, redox-sensitive peptides of WT leaves, 74 cysteines in 71 different proteins were in a more reduced state in the VC-treated than in nontreated plants, whereas 49 cysteines in 47 different proteins were more oxidized in the VC-treated than in nontreated plants (Table S5). Furthermore, of the 87 VC-responsive, redox-sensitive peptides of *ntrc* plants, 56 cysteines in 44 different proteins were in a more oxidized state in the VC-treated than in nontreated plants, whereas 44 cysteines in 41 different proteins were more reduced in VC-treated than in nontreated plants (Table S6).

Predicted locations of the VC-responsive, redox-sensitive proteins of WT and *ntrc* plants obtained using the SUBA4 *Arabidopsis* protein

subcellular localization database included almost all cellular compartments, but the highest proportions had predicted plastidial, nuclear, and mitochondrial locations (Tables S5 and S6 and Figure S8). Using DiANNA software (http://clavius.bc.edu/~clotelab/DiANNA/), 50% and 60% of the redox-sensitive peptides of WT and *ntrc* plants, respectively, were predicted to form intramolecular disulfide bonds (Tables S5 and S6). It should be noted that formation of disulfide bridges represents only one possibility of thiol modifications (Buchanan & Balmer, 2005; Couturier et al., 2013), and other modifications such as glutathionylation, nitrosylation, and oxidation to sulfenic acid are also chemically reversible by DTT, which was used to reduce oxidized thiols before the reaction with thiol-reactive IAM. Therefore, some of the VC-promoted reversible thiol modifications identified in this study could be due to any of those types.

Using broad characterizations outlined by the MapMan tool (Thimm et al., 2004; http://gabi.rzpd.de/projects/MapMan/), the VC-responsive, redox-sensitive proteins were assembled into functional groups. Most were assigned to protein, RNA, signalling, and stress response functional categories (Tables S5 and S6 and Figure 5). Notably, VC exposure resulted in reduction of cysteine residues of several CBC enzymes (e.g., cFBP1, PRK, and SBP), proteins involved in photochemical reactions of photosynthesis (e.g., FNRL and PSAN), and PrxQ in WT leaves (Table S5 and Figure 5a). In addition, fungal VC exposure of *ntrc* plants resulted in oxidation of cysteine residues of proteins involved in photochemical reactions of photosynthesis (e.g., FNR1, PSAN, and PSBO-1) and PrxQ (Table S6 and Figure 5b). All these cysteine residues are highly conserved throughout land plants and algae (Figure S9).

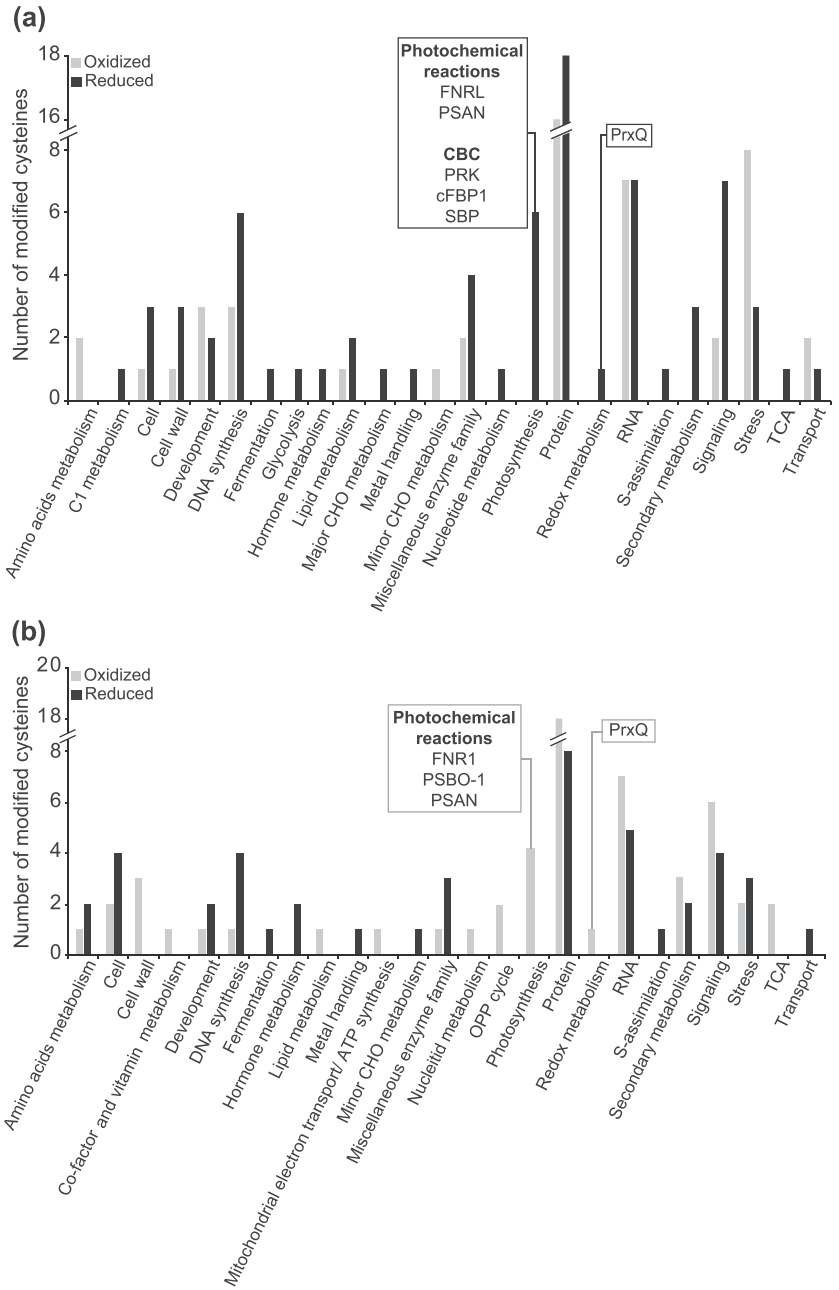

FIGURE 5 *Alternaria alternata* volatile compounds (VCs) promote global reduction and oxidation of the thiol redox proteomes of wild-type leaves and *ntrc* leaves, respectively. The graphics represent functional categorizations of VC-responsive redox sensitive proteins in (a) wild-type and (b) *ntrc* plants, sorted according to putative functional categories assigned by MapMan software. Numbers of oxidized and reduced cysteines in each categorical group are indicated by grey and black bars, respectively. Proteins discussed in the text are boxed

4 | DISCUSSION

4.1 | NTRC is an important mediator of plant responses to microbial VCs

VCs emitted by *A. alternata* and other microbial species had weak stimulatory effects on growth and development of *ntrc* plants (Figures 1a,b and S3), strongly indicating that NTRC plays an important role in plant responses to microbial VCs. The weak response of *ntrc* plants to fungal VCs cannot be ascribed to altered starch metabolism because growth responses of the starch-deficient *aps1* and the starch-excess *gwd* plants were comparable with those of WT plants (Figure S1).

Unlike in WT plants, chlorophyll content did not increase upon fungal VC treatment in *ntrc* plants (Figure 3a), indicating that NTRC is a major determinant of chlorophyll biosynthesis in VC-exposed plants and no other reductant system(s) can functionally replace NTRC in promoting photosynthetic pigment production under VC exposure. Also, Φ_{NPQ} did not vary and *qP* did not increase upon fungal VC treatment in *ntrc* plants (Table 2), indicating that, unlike in WT plants, VCs do not improve the efficiency of light use in *ntrc* leaves and, hence, do not improve photosynthetic CO_2 fixation. This inference was supported by the finding that fungal VCs did not alter V_{cmax}, J_{max}, and *TPU* values of *ntrc* plants but increased those of WT plants (Table 2). These observations suggest that NTRC is a major mediator of the plants' responses to VCs, probably due to its regulatory action on photosynthesis-related mechanisms.

Previous reports have shown that microbial VCs can induce changes in root development (Ditengou et al., 2015; García-Gómez et al., 2019; Garnica-Vergara et al., 2016; Molina-Favero, Creus, Simontacchi, Puntarulo, & Lamattina, 2008). Furthermore, Kirchsteiger et al. (2012) reported that NTRC is involved in LR formation in *Arabidopsis* seedlings by unidentified mechanisms. Here, we found that, unlike in WT plants, VCs did not promote formation of LR in *ntrc* plants (Figure 1c), strongly indicating that NTRC is an important determinant of LR formation in response to *A. alternata* VCs. CKs and ABA are major regulators of photosynthesis, growth, and development (Cortleven & Valcke, 2012; Kieber & Schaller, 2014; Moore et al., 2003; Rolland, Baena-Gonzalez, & Sheen, 2006). In WT plants, microbial VCs promote reduction of ABA levels and increases in plastidial CK contents (Sánchez-López, Baslam, et al., 2016; Zhang et al., 2008), which are accompanied by changes in the expression of ABA- and CK-responsive genes (Sánchez-López, Bahaji, et al., 2016; Sánchez-López, Baslam, et al., 2016; Zhang et al., 2008). *Arabidopsis* mutants with CK deficiency, and/or reduced endogenous ABA and CK receptor sensitivity, weakly respond to microbial VCs (Sánchez-López, Baslam, et al., 2016; Zhang et al., 2008), indicating that plant responses to microbial VCs involve endogenous ABA and CK signalling. NTRC is a good reductant of CHLI (Pérez-Ruiz et al., 2014), which together with the putative ABA receptor CHLH (Du et al., 2012; Shang et al., 2010; Shen et al., 2006; Wu et al., 2009) is involved in ABA signalling in processes such as stomatal movement, seed development, and seedling growth (Du et al., 2012; Tsuzuki et al., 2011). Moreover, NTRC is an important determinant of posttranslational regulation of CHLI expression, as impairment of the NTRC-mediated redox regulation of CHLI destabilizes the protein (Pérez-Ruiz et al., 2014). Notably, here we found that responses in expression of ABA-responsive genes to VC treatment were substantially weaker in *ntrc* plants than in WT plants (Figure 2). This suggests that NTRC affects ABA signalling in VC-exposed plants through its action on CHLI redox status. Therefore, the nonresponsiveness of ABA-responsive genes in *ntrc* plants to fungal VCS could be due, at least partly, to the lack of active CHLI. As responses in expression of CK-responsive genes to VC treatment were weaker in *ntrc* plants than in WT plants (Figure 2), it is tempting to speculate that NTRC participates in CK signalling in VC-exposed plants through unidentified mechanisms involving regulation of the redox status of proteins that mediate this signal pathway. Clearly, further experiments are needed to test this hypothesis.

4.2 | Weak responses of *ntrc* plants to microbial VCs are not due to ROS over-accumulation

Photosynthesis involves transport of electrons in the presence of oxygen and, thus, is a major source of ROS. When high levels accumulate under stress conditions in which PET rates exceed photosynthetic capacity, ROS over-accumulation may cause photooxidative damage (Apel & Hirt, 2004; Stenbaek et al., 2008). Microbial VCs enhance photosynthetic ETR in *ntrc* plants (Figure 3c) but have no effect on A_n (Figure 3b), thereby creating conditions that promote ROS production. In *Arabidopsis*, NTRC is a good reductant of the two H_2O_2 detoxifying 2-Cys Prxs, hence suggesting it plays an important role in preventing excessive ROS accumulation (Kirchsteiger et al., 2009; Pérez-Ruiz et al., 2006; Puerto-Galán et al., 2015; Pulido et al., 2010). Notably, as in WT plants, VCs strongly promoted photosynthesis and growth of *ntrc–Δ2cp* plants (Figure 4a–d). Furthermore, leaves of VC-treated and nontreated WT, *ntrc*, *Δ2cp*, and *ntrc–Δ2cp* plants accumulated comparable ROS levels (Figure S7). These findings indicate that *ntrc* plants' weak responses to VCs are not due to ROS over-accumulation as a consequence of 2-Cys Prxs inactivation. They also indicate that the stimulatory effect of microbial VCs on plant performance requires an appropriate chloroplast redox homeostasis through mechanisms wherein the NTRC–2-Cys–Prx tandem plays a central function.

4.3 | *A. alternata* VCs promote changes in the thiol redox-proteome of WT plants that could account for the observed plants' responses

The redox proteomic analyses conducted here provide an in-depth report of thiol-based redox proteins that are responsive to microbial VCs in plants. Our results show that microbial VCs promote global reduction of the thiol redox proteome of WT leaves, especially of proteins involved in cell wall metabolism, nucleotide metabolism, photosynthesis, protein synthesis and processing, redox metabolism, secondary metabolism, and signalling (Figure 5), which could partly explain the stimulatory growth responses of plants to VCs. Notably,

microbial VCs promote global changes in the redox status of proteins distributed in all cellular compartments but especially in plastids, mitochondria, and the nucleus (Figures 5 and S8 and Tables S5 and S6), indicating that interorganellar redox crosstalk is involved in maintenance of whole cells' redox balance. The results highlight a redox switching mechanism in VC signalling and provide additional confirmation of the hypothesis that, as illustrated in Figure 6, microbial VC signalling operates at multiple (including transcriptional, translational, and posttranslational) levels (García-Gómez et al., 2019; Sánchez-López, Baslam, et al., 2016).

Redox regulation is inextricably associated with the PET chain and CBC enzymes in plants. Besides the earliest studied CBC enzymes (e.g., cFBP1, PRK, SBP, and GAPDH), recent biochemical and proteomic studies have provided evidence that all CBC enzymes may undergo redox regulation through multiple redox posttranslational modifications (Michelet et al., 2013). We found that fungal VCs promote reduction of cysteine residues of CBC enzymes in WT plants (e.g., Cys154 of cFBP1, Cys150 of SBP, and Cys295 of PRK; Figure 5a and Table S5). Although highly conserved throughout land plants and algae (Figure S9), these cysteine residues are not located in the proposed regulatory Trx redox or catalytic domains of cFBP1, PRK, and SBP (Brandes, Larimer, & Hartman, 1996; Chiadmi, Navaza, Miginiac-Maslow, Jacquot, & Cherfils, 1999; Dunford, Durrant, Catley, & Dyer, 1998; Villeret, Huang, Zhang, Xue, & Lipscomb, 1995). However, it should be noted that, besides the structural role of disulfide bonds, redox changes in proteins' cysteine residues can have diverse effects. They can participate in regulatory, protective, catalytic, and signalling mechanisms by influencing subcellular localization or protein–protein interactions and promoting conformational changes that affect the modified proteins' biological activities. Therefore, the redox status of Cys154 of cFBP1, Cys150 of SBP, and Cys295 of PRK could play roles in the respective enzymes' stability, subcellular localization, and/or formation of protein complexes in response to varying environmental conditions. In addition, reduction of some of these cysteine residues may be involved in the VC-promoted enhancement of photosynthesis in WT plants.

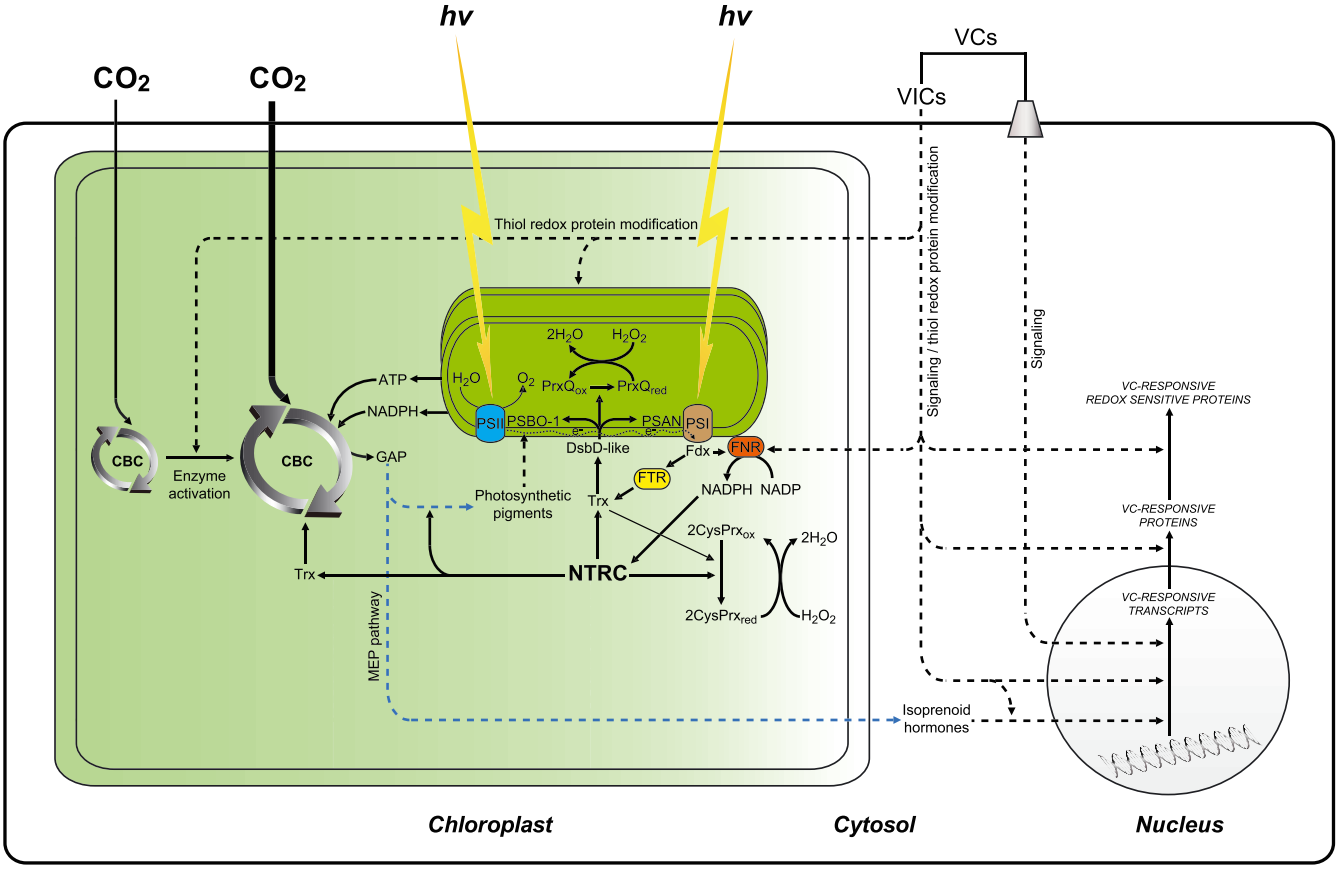

FIGURE 6 Suggested model for regulation of plants' responses to microbial volatile compounds (VCs) involving posttranslational thiol redox proteome changes and NADPH-dependent thioredoxin reductase C (NTRC). According to this model, interactions between VCs and unidentified plasma membrane receptors generate signals that rapidly promote changes in expression of VC-responsive genes. Additionally, and/or alternatively, reactive VIC-penetrating cells may induce thiol redox modifications of proteins and/or signalling reactions that promote reductive activation of photosynthesis-related proteins (e.g., FNR1, cFBP1, SBP, PRK, PSBO-1, and PSAN). The resulting augmentation of photosynthetic activity enhances synthesis of GAP, which enters the MEP pathway, fuelling production of isoprenoid hormones that initiate a cascade of redox-regulated signalling reactions resulting in changes in the expression of genes whose translation is subject to redox regulation. Reductive activation of enzymes involved in photochemical reactions enhances NADPH production, thereby influencing whole cells' redox balance, reduction of NTRC and NTRC's target enzymes involved in processes such as synthesis of photosynthetic pigments, H_2O_2 detoxification, and CBC

The redox proteomic analysis of WT plants performed in this study also showed that VCs promote reduction of cysteine residues of two proteins involved in photochemical reactions of photosynthesis: PSAN and FNRL. These cysteine residues (e.g., Cys155 of PSAN and Cys141 of FNRL) are highly conserved in land plants and algae (Figure S9), highlighting the importance of their roles throughout a very long period of evolutionary history. PSAN is essential for efficient interaction between plastocyanin and P700 reaction centre of the PSI complex and thus participates in optimization of electron flow (Haldrup, Naver, & Scheller, 1999). FNRL is a FAD-containing $NADP^+$ oxidoreductase likely involved in PET (Koskela et al., 2018). It is tempting to speculate that VC-promoted ETR enhancement (Figure 3c) is at least partly due to reductive activation of PSAN and FNRL.

4.4 | The *ntrc* mutant shows global oxidation of its redox proteome, especially photosynthesis-related proteins

In contrast to WT plants, fungal VCs promoted global oxidation of the redox proteome of leaves of *ntrc* plants, especially of proteins related to cell wall metabolism, nucleotide metabolism, photosynthesis, protein synthesis and processing, redox metabolism, secondary metabolism, and signalling (Figure 5 and Table S6), which could partly explain the weak responses of *ntrc* plants to VCs. As microbial VC-promoted global oxidation of the redox-proteome involved proteins distributed in all subcellular compartments in *ntrc* plants (Figure S8), NTRC conceivably plays an important role in maintaining whole cell's redox homeostasis in VC-exposed plants.

Fungal VCs promoted oxidation of Cys155 of PSAN in *ntrc* plants (Table S6), which became more reduced in WT plants exposed to fungal VCs (see above). This indicates that PSAN is subject to redox regulation in VC-exposed plants through mechanisms involving NTRC. In addition, fungal VCs promoted oxidation of Cys114 of one of the two PSBO isoforms (e.g., PSBO-1) in *ntrc* plants (Table S6), which is a component of the PSII involved in water oxidation and formation of molecular oxygen (Murakami et al., 2005). Like Cys155 of PSAN, Cys114 of PSBO-1 is highly conserved in land plants and algae (Figure S9). It is tempting to speculate that weak VC-promoted ETR enhancement in *ntrc* plants (Figure 3c) could be at least partly due to reduced electron flow as a consequence of PSAN and PSBO-1 oxidation.

Microbial VCs also promoted oxidation of Cys178 and Cys183 of FNR1, both of which are highly conserved in land plants and algae (Figure S9). FNR1 is one of the two isoforms of leaf Fdx:$NADP^+$(H) oxidoreductase (FNR) that oxidize the final reduced product of PET, thereby generating the NADPH required for stromal redox regulation, reduction of NTRC and its target proteins, and enzymes involved in diverse metabolic pathways, including the CBC (Ceccarelli, Arakaki, Cortez, & Carrillo, 2004). Leaf FNR is also implicated in cyclic electron transfer around PSI, which generates a proton gradient across the thylakoid membrane, resulting in ATP production (Johnson, 2005). FNR1-lacking plants grow slowly and accumulate low levels of chlorophyll (Lintala et al., 2007). Notably, Cys178 is essential for FNR1 enzymatic activity (Aliverti et al., 1993). Thus, NTRC expression seems to be necessary to prevent FNR1 oxidation in VC-exposed plants. Moreover, the reduced FNR1 activity might contribute to the lack of enhancement of photosynthetic capacity, reduction of the NADPH/NADP ratios, and global oxidation of the redox proteome in VC-exposed *ntrc* plants (Tables 2 and S6 and Figures 3 and S6).

In vitro, PrxQ is a good target of NTRC (Yoshida & Hisabori, 2016). In WT plants, fungal VCs promoted reduction of Cys116 of this Prx (Table S5 and Figure 5a), which is conserved throughout land plants and algae (Figure S9). In contrast, fungal VCs promoted oxidation of the same cysteine residue in *ntrc* plants (Table S6 and Figure 5b), suggesting that PrxQ is subject to redox regulation in VC-exposed plants to prevent excessive H_2O_2 accumulation through mechanisms involving NTRC as schematically illustrated in Figure 6. Both PSAN and PrxQ are luminal proteins (Petersson, Kieselbach, García-Cerdán, & Schröder, 2006), although there are indications that PrxQ may associate with PSII in the grana stacks (Lamkemeyer et al., 2006) or thylakoids on the stromal side (Rouhier et al., 2004). Putative interactions between NTRC, located in the stroma, and PSAN and PrxQ might occur through the DsbD-like transmembrane pathway for disulfide–thiol exchange, which accepts electrons from stromal Trx-m and transfers these reducing equivalents to luminal proteins (Karamoko, Gabilly, & Hamel, 2013; Motohashi & Hisabori, 2006; Figure 6).

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AUTHOR CONTRIBUTIONS

K. A., M. B., and J. P.- R. designed the experiments and analysed the data; K. A., M. B., L. U., A. M.S.- L., F. J. M., A. B., G. A., P. G.- G., E. B.- F., N. D. D., J. F. H., and K. K. performed most of the experiments; L. S., K. D., T. M., F. J. C., and J. P.- R. supervised the experiments; K. A., M. B., and J. P.- R. wrote the article with contributions from all the authors; J. P.- R. conceived the project and research plans;

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SUPPORTING INFORMATION

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

Figure S1. Growth responses of starch excess (*gwd*) and starch deficient (*aps1*) mutants to fungal VCs are comparable to those of WT plants.

Figure S2. External root phenotypes of WT and *ntrc* plants cultured in the absence or continuous presence of VCs emitted by adjacent *A. alternata* cultures for one week. Scale bars = 1 mm.

Figure S3. *ntrc* plants weakly respond to VCs emitted by phylogenetically diverse microorganisms.

Figure S4. VCs emitted by *A. alternata* promote augmentation of the levels of CKs in leaves of WT and *ntrc* plants.

Figure S5. Chlorophyll-a fluorescence images of WT and *ntrc* plants cultured in the absence or presence of VCs emitted by adjacent *A. alternata* cultures for three days.

Figure S6. Levels of (a) NADP and (b) NADPH in leaves of WT and *ntrc* plants cultured in the absence or continuous presence of VCs emitted by adjacent *A. alternata* cultures for one week.

Figure S7. ROS staining of WT and *ntrc* plants cultured in the absence or presence of five days of adjacent *A. alternata* cultures.

Figure S8. Categorization of VC-responsive redox-sensitive proteins in (a) WT and (b) *ntrc* plants according to their subcellular localization.

Figure S9. Alignment of mature forms of FNR1, FNRL, PSAN, PSBO-1, PRK, SBP, cFBP1 and PrxQ from indicated land plants (e.g. *Arabidopsis thaliana*, *Oryza sativa*, *Spinacia oleracea*, *Triticum aestivum* and *Zea mays*) and algae (e.g. *Chlamydomonas reinhardtii).*

Table S1. Primers used in qRT-PCR

Table S2. CK contents in leaves of WT and *ntrc* plants cultured in solid Murashige and Skoog medium in the absence or continuous presence of VCs emitted by adjacent *A. alternata* cultures for 3 days.

Table S3. List of cysteine-containing peptides identified in the redox-proteomic study of WT plants cultured in the absence or continuous presence of VCs emitted by adjacent *A. alternata* cultures for 3 days.

Table S4. List of cysteine-containing peptides identified in the redox-proteomic study of *ntrc* plants cultured in the absence or continuous presence of VCs emitted by adjacent *A. alternata* cultures for 3 days.

Table S5. List of VC-responsive redox-sensitive peptides in leaves of WT plants identified in the redox-proteomic analysis.

Table S6. List of VC-responsive redox-sensitive peptides in *ntrc* plants identified in the redox-proteomic analysis.

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