Arabidopsis thimet oligopeptidases are redox-sensitive enzymes active in the local and systemic plant immune response

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Upon pathogen infection, receptors in plants will activate a localized immune response, the effector-triggered immunity (ETI), and a systemic immune response, the systemic acquired response (SAR). Infection also induces oscillations in the redox environment of plant cells, triggering response mechanisms involving sensitive cysteine residues that subsequently alter protein function. Arabidopsis thaliana thimet oligopeptidases TOP1 and TOP2 are required for plant defense against pathogens and the oxidative stress response. Herein, we evaluated the biochemical attributes of TOP isoforms to determine their redox sensitivity using ex vivo Escherichia coli cultures and recombinant proteins. Moreover, we explored the link between their redox regulation and plant immunity in wild-type and mutant Arabidopsis lines. These analyses revealed that redox regulation of TOPs occurs through two mechanisms: (1) oxidative dimerization of full-length TOP1 via intermolecular disulfides engaging cysteines in the N-terminal signal peptide, and (2) oxidative activation of all TOPs via cysteines that are unique and conserved. Further, we detected increased TOP activity in wild-type plants undergoing ETI or SAR following inoculation with Pseudomonas syringae strains. Mutants unable to express the chloroplast NADPH-dependent thioredoxin reductase C (NTRC) showed elevated TOP activity under unstressed conditions and were SAR-incompetent. A top1top2 knockout mutant challenged with P. syringae exhibited mis-regulation of ROS-induced gene expression in pathogen-inoculated and distal tissues. Furthermore, TOP1 and TOP2 could cleave a peptide derived from the immune component ROC1 with distinct efficiencies at common and specific sites. We propose that Arabidopsis TOPs are thiol-regulated peptidases active in redox-mediated signaling of local and systemic immunity.

Hindering pathogen proliferation and tissue damage is a common challenge for organisms across the tree of life. Plants respond to pathogen infection by activating localized and systemic defense responses. Intracellular immune receptors, such as RPS2, initiate the effector-triggered immune response (ETI) by recognizing effectors such as Pseudomonas syringae’s AvrRpt2 at the site of pathogen infection (1). Following this localized immune response, organs distal from the infection site activate the systemic acquired resistance (SAR) (2), a type of systemic immunity that confers long-lasting protection to secondary infections (3).

As a central element in the plant interaction with pathogens, the redox state in the plant cells is dynamic and tightly controlled (4). The immune response activation relies on the rapid accumulation of reactive oxygen species (ROS) in multiple subcellular compartments. Oxidants such as hydrogen peroxide (H₂O₂) are generated metabolically and enzymatically by, for example, NADPH-dependent oxidases and may accumulate within precise spatial and temporal parameters during an immune response (5). The sudden ROS upsurge postinfection, called the “oxidative burst,” causes redox modifications in proteins required to activate local and systemic immunity and the programmed cell death pathways (6, 7). Protein thiols toggling between reduced and oxidized states constitute a universal redox-sensing mechanism that modulates host signaling in response to cellular redox potential (8) and critical for pathogenesis (9). Coordination among diverse subcellular compartments, including the cytosol, chloroplast, and mitochondria, has been recognized as essential for cellular redox homeostasis. Reductive systems, including glutathione (GSH), NAD/NADP, and ascorbate, function across multiple cellular compartments and constitute an essential conduit for ROS processing and redox signaling (10).

Although immune-associated redox processes have been studied since the 1970s (11, 12), and numerous studies since have demonstrated their essentiality to immune signal transduction (10–15), our knowledge of the redox biology underlying plant immunity is still basic (16–18). Redox proteomics has provided evidence of hundreds of redox-sensitive protein thiols; however, few are associated with a specific function (19, 20). One well-studied redox-modulated immune component is the transcription cofactor NPR1, which is converted by

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thioredoxins from an inactive oligomer to active monomers to induce SAR via a pathway controlled by the plant defense hormone salicylic acid (SA) (21).

Recent studies highlight the interconnectivity between cytosolic and organellar protein homeostasis networks in metazoan’s response to oxidative stress (22, 23). Our understanding of the plant proteostasis networks and their roles in immunity and redox signaling stress is emerging (24, 25). In this context, we study thimet oligopeptidases (TOPs), a family of evolutionarily conserved peptidases with a metal-binding His-Glu-Xaa-Xaa-His (HEXXH) active site motif (26). Plant TOPs are encoded by multiple genes translated into proteins with medium-to-low similarity to metazoan TOPs (27, 28). Arabidopsis thaliana TOP1, or organellar oligopeptidase (OOP), contains an N-terminal dual-localization signal (transit) peptide of ~100 amino acids for transport into chloroplasts and mitochondria (28–30). Proteomic studies have also detected cytosolic full-length TOP1 (31). Organellar TOP1 is a component of the protein import pathways with roles in processing transit peptides (29). The closely related cytosolic TOP2 (CyOOP) was associated with the degradation of oxidized peptide products released from the 20S proteasome (32). TOP1 lacking the predicted target sequence (herein named ΔTOP1) was crystallized in a closed conformation resembling the Escherichia coli dipeptidyl carboxypeptidase (29). TOP2 was crystallized in an open conformation resembling metazoan THOPs (33).

We previously demonstrated that TOP1 and TOP2 are components of plant immune pathways necessary to activate ETI and sustain an optimal oxidative burst. We showed that SA bound and inhibited TOP1 activity and facilitated its dimerization, whereas SA interacted with ΔTOP1 and TOP2 with much lower affinity and had a lesser impact on their activity (28, 34). A top1top2 knockout mutant had increased susceptibility to pathogen infection, abnormal oxidative burst, and defective levels of proteome oxidation during ETI (25, 28). Biochemical studies of TOPs suggested a possible redox regulation of their functions (34). These studies collectively confirmed the cooperative roles of A. thaliana TOPs in immunity and oxidative stress and highlighted apparent differences in TOPs biochemical characteristics. Here, we undertake a detailed comparative investigation of the redox sensitivity of A. thaliana TOPs. We address the roles of TOPs cysteine residues for TOPs self-interaction and enzymatic activity ex vivo and in vitro and the identity of redox-sensitive cysteines. Further, we assess TOPs activity in the context of plant immune response, evaluate an oxidoreductase’s role as a TOPs regulator, and test TOPs cleavage specificity on a peptide derived from a critical immune regulator.

Results

Structural characteristics of TOP1 and TOP2 influence their physicochemical properties

We hypothesized that the structural characteristics of A. thaliana TOPs determine their biochemical properties. To gain insight into potential differences among TOP isoforms, we generated a three-dimensional (3D) model of full-length TOP1 via homology modeling (35) using as a template the structure of the organellar TOP1 lacking the N-terminal signal peptide sequence (29). We compared the TOP1 model with Δ58TOP1 (29) and TOP2 (33) structures to assess the location and conservation of Cys residues (Fig. 1A). Three Cys residues situated in the peptidase domain are conserved – C548/460, C611/523, and C699/611 in TOP1/TOP2. C29, C42, and C52 are located in the signal peptide of TOP1 and thus unique for the full-length TOP1, whereas C405 is unique for TOP2 (Fig. 1B). Measurement of Cys-to-Cys distances in ΔTOP1, TOP2, and the modeled TOP1 showed that Cys residues are positioned within distances larger than ~2 Å required for forming intramolecular disulfide bonds (36). Notably, C29 and C52 in TOP1’s signal peptide are predicted to be solvent-exposed, whereas C405 of TOP2 has the lowest buried coefficient among its cysteines, suggesting a potential for the redox regulation of these solvent-exposed residues (Fig. S1).

A characteristic feature of TOPs is the hexapeptide zinc-binding motif (HEXXGH). In the plant TOPs, as in other zinc metallopeptidases, His, His, and Glu residues coordinate with Zn2+ to form hydrogen bonds with acidic amino acid residues (Glu or Asp) (Fig. 1C). The binding site’s geometry in many bacterial metallopeptidases allows for Zn2+ substitution, with subsequent effects in some cases, on other properties such as protein stability (37, 38). To determine the metal ion-binding properties of TOPs, recombinant TOP1-His and TOP2-His were purified from E. coli cultures. The 6xHis tags were removed by thrombin cleavage before the incubation of TOP1 and TOP2 with metal ions and analysis by inductively coupled mass spectrometry. We found that TOP1 preferentially bound Zn2+ (2.7 mg/L) and had lower affinities for Cu2+, Co2+ (~0.5 mg/L), and Mn2+ (<0.01 mg/L) (Fig. 1D). Comparatively, TOP2 had a more permissive ion-binding ability; TOP2 bound Co2+ (3.4 mg/L), Zn2+, Mn2+, and Cu2+ (each at ~1.5–2 mg/L) (Fig. 1D). Altogether, differences in TOPs primary and tertiary structures suggest distinct modes of regulation.

Oxidation promotes the dimerization of TOP1 but not of TOP2

We hypothesized that the observed structural differences between TOP1 and TOP2 impact redox behaviors such as self-interaction. We first tested this hypothesis using an ex vivo redox titration system adapted from (39). E. coli cultures expressing recombinant TOPs were treated with defined ratios of GSH/GSSG and DTTred/DTTox solutions to yield a range of reducing and oxidizing environments (~360 to ~100 mV) (Fig. 2A and Supplemental Methods). Ten hours posttreatment (hpt), total protein extracts were separated on native PAGE and subjected to immunoblotting using anti-His antibodies. Following GSH/GSSG treatments, the anti-His antibody detected monomeric TOP1 and higher-MW bands in total extracts and a control purified TOP1 sample (Fig. 2A, left). The monomeric and high-MW bands were also detected in extracts from cells subjected to the full range of redox treatments with GSH/GSSG and DTTred/DTTox (Fig. S2A).
A similar analysis of TOP2-expressing cells treated with GSH or GSSG detected solely monomeric TOP2 in total extracts and a purified TOP2 sample (Fig. 2A, right). Notably, in extracts run on a 3–12% native PAGE, the anti-His antibody detected a slower-migrating TOP2 band (Fig. S2B), which may be dimeric TOP2. We tested this possibility by analyzing total extracts from DTTred- and GSSG-treated TOP2-expressing cells using 2D electrophoresis; only TOP2 monomers were detected under both conditions (Fig. S2D).

The self-interaction behavior of TOPs under diverse redox conditions was further analyzed using purified recombinant proteins and size-exclusion HPLC (SEC). Protein size markers, including bovine serum albumin (BSA) known to dimerize (40), were run through the size-exclusion chromatography (SEC) column, and a regression line was plotted using the retention times (Rt) and MWs (Fig. S2C). Purified recombinant TOP1, ∆SPTOP1, and TOP2 were treated with DTTred, DTTox, GSG, or GSSG and run through the SEC column. We found that TOP1 eluted mainly as two peaks, one prominent monomeric peak and one smaller corresponding to dimeric TOP1, alongside a low-abundance trimeric peak (Fig 2B and Fig. S2C). A quantitative assessment of peak area showed that the TOP1 M/D ratio remained constant post-DTTred and -DTTox incubations (~7/1 under both conditions); however, the M/D ratio decreased markedly postglutathione treatments (3/1 and 2/1 post-GSH and -GSSG, respectively) (Fig. 2B, lower right inset). TOP1 monomeric and higher-MW structures were also visible in the SEC-analyzed TOP1 samples separated on native PAGE and stained with a generic protein stain (Fig. 2B, upper right inset). In contrast to the full-length isoform, ∆SPTOP1 was detected solely as a monomer following reductive (DTTred) and oxidative (GSSG) treatments, as shown by SEC analysis (Fig. 2C) and anti-His immunoblotting (Fig. 2C, right inset). TOP2 analysis revealed that incubation with DTTred, DTTox, GSH, or GSSG failed to induce its dimerization; solely monomers were detected by SEC analysis (Fig. 2D) and an anti-His immunoblot of the SEC-analyzed samples (Fig. 2D, inset).

Mapping cysteine residues involved in TOP1 intermolecular cross-linking

So far, the results indicated that TOPs exist predominantly as monomers ex vivo and in vitro and brought forth the prospect that the signal peptide of TOP1 is required for its dimerization. We used site-directed mutagenesis to generate mutant TOPs with individual cysteines changed to redox-
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Insensitive alanine. Recombinant proteins were expressed in E. coli (Fig. S3A), purified, and treated with oxidizing agents prior to SEC analysis; untreated purified proteins served as controls.

Following GSSG treatment, TOP1C29A, TOP1C52A, and TOP1C611A showed smaller dimeric peaks than TOPC42A, TOPC548A, or TOP1C699A (Fig. 3A). Moreover, unlike the native TOP1, untreated TOP1C52A, TOP1C611A, and TOPC42A mutants eluted solely as monomers. TOP1C29A consistently showed low peak intensities in SEC, suggesting a more general effect of this mutation on the monomer stability. A quantitative assessment of the peaks showed that C52 and C611 had the highest impact on TOP1 dimerization (Fig. 3B).

SEC analysis of the GSSG-treated TOP2 mutants – C405A, C460A, C523A, and C611A – determined that none of the individual mutations affected TOP2 monomerization, as shown by the elution of all mutant proteins in monomer-size peaks (Fig. 3C). TOP2 mutant proteins were also analyzed ex vivo using E. coli cultures expressing individual mutants treated with GSH or GSSG. The anti-His antibody detected solely monomeric TOP2 in the respective total extracts; the monomeric signal intensity was weaker in mutants than the native TOP2, suggesting that these mutations may impact monomer accumulation or stability (Fig. S3C).

Exposure to oxidizing conditions might facilitate TOP1 dimerization via disulfides. We performed LC-MS to identify cysteines involved in TOP1 dimerization. Native PAGE bands from TOP1-expressing E. coli cells treated with GSSG were subjected to in-gel digestion with trypsin and chymotrypsin and analyzed. Reduction and alkylation of cysteines, typically employed in bottom-up proteomics, were omitted to preserve disulfide bonds. A protein database search was used first to confirm TOP1 detection (Fig. S3C). The LC-MS data were manually interrogated for ions corresponding to the charge states of theoretical,
disulfide-bound dipeptides. This strategy revealed three disulfide-bound dipeptides, showing intermolecular connectivity between C29–C52 (Fig. 4A), C42–C52 (Fig. 4B), and C52–C52 (Fig. 4C). These matched theoretical dipeptide masses with <2 ppm mass error. These dipeptides were not detected in similarly processed monomeric TOP1 samples, providing further confidence in these peptide assignments.

Figure 3. Redox-sensitive cysteine residues in TOP1 signal peptide and peptidase domain control TOP1 homodimerization. A, size-exclusion chromatography (SEC) analysis of purified TOP1 Cys-to-Ala mutants in the absence of redox treatments (Ctrl) and after incubation with GSSG. The chromatograms show the position of monomeric (M), dimeric (D), and trimeric (T) peaks (n = 2). B, peak area quantification for TOP1 samples analyzed in A showing percentage of TOP1 dimers in one representative experiment. C, SEC analysis of purified TOP2 Cys-to-Ala mutants after incubation with GSSG (n = 2).
Redox-sensitive cysteine residues control TOP1 and TOP2 catalytic activity

Changes in the redox state of protein thiols may impact TOPs activity level. To determine the effect of the ambient redox potential on TOP activity, purified TOP1 and TOP2 were incubated in solutions containing defined ratios of DTT_{red}/DTT_{ox} and GSH/GSSG. Treated TOPs were mixed with the TOP-specific fluorogenic peptide (Mca-PLGPK(DNP)-OH) (41). We found that oxidation markedly augmented TOP1 activity, assessed by measuring the cleaved product’s fluorescence (Fig. 5A). TOP1 activity was maintained at a low and constant level for the −360 to −322 mV range but gradually increased in the −220 to −100 mV range. The midpoint redox potential (E_m) was estimated from the nonlinear best fit of the fraction of reduced protein versus the ambient redox potentials (42, 43). We estimated TOP1’s E_m at −199.5 ± 1.4 mV at pH 7.0 (95% confidence interval, −203 to −195.9 mV) when fitting the Nernst equation for a single, two-electron redox reaction (Fig. S5). Similar to TOP1, TOP2 activity was also increased by oxidation; on average, TOP2 was 3-fold more active post-GSH/GSSG compared with DTT_{red}/DTT_{ox} treatments; unlike TOP1, TOP2 activity gradually decreased with increasing oxidation within the E_m range of both types of treatments, although it retained over 85% activity at strongly oxidizing E_m values (Fig. 5B). Overall, the results indicate that oxidation augments TOPs activity.

Next, to determine the cysteine residues necessary for TOPs oxidative activation, we compared the oxidized (GSSG-treated) and reduced (DTT_{red}-treated) mutants and native TOPs. GSSG treatment augmented TOP1 native and mutant proteins. However, we measured significantly lower activity levels for ΔSPTOP1 and four of the Cys-to-Ala mutants (C52A, C548A, C611A, and C699A), suggesting that multiple cysteines contribute to TOP1 oxidative activation (Fig. 5C). Measurements of native and mutant TOP2 proteins verified the activity enhancement of the oxidized native TOP2 and revealed that two mutations had a significant impact: the C405A mutation abolished, and C611A impaired TOP2 oxidative activation (Fig. 5D).

TOP activity increases during the plant immune response and is under the regulation of the NTRC oxidoreductase

Our observations herein pointed to oxidative activation as a common characteristic of plant TOPs. We previously demonstrated that TOP1 and TOP2 are required for the optimal activation of the oxidative burst and ETI triggered by...
infection with *P. syringae* DC3000 AvrRpt2 (PstAvrRpt2) (44, 45). Thus, TOP enzymatic activities might be required and influenced by the cellular redox homeostasis fluctuations characteristic of the plant immune response. We determined TOP activity in wild-type Col-0 plants undergoing PstAvrRpt2-triggered ETI and compared them to buffer-infiltrated controls (Fig. 6A). TOP activity on the fluorogenic TOP substrate was measured in total protein extracts (100 μg) from leaves collected 48 h postinoculation (hpi) with PstAvrRpt2. Substantially higher (approximately 3-fold) amounts of cleaved substrate accumulated in reactions with extracts from pathogen-inoculated than control leaves (Fig. 6B).

A localized infection with PstAvrRpt2 induces a generalized immune response throughout the plant—the SAR (2). We tested TOP activity in systemic leaves from plants challenged with a localized inoculation of PstAvrRpt2 and buffer-only infiltrations as control (Fig. 6A). We measured a 1.5-fold increase in TOP activity in systemic leaves from PstAvrRpt2-challenged plants compared with controls at 48 hpi (Fig. 6C).

To ascertain that SAR was triggered in Col-0 by the localized PstAvrRpt2 infiltration, systemic leaves were inoculated with the virulent pathogen PstDC3000 at 48 h postchallenge with PstAvrRpt2 or buffer, followed by measurements of PstDC3000 growth (Fig. 6D). As expected, a significant decrease in PstDC3000 growth occurred in plants challenged with PstAvrRpt2 compared with buffer-infiltrated plants at 48 hpi, indicating SAR activation. Control measurements of PstDC3000 titers immediately after leaf inoculation showed no significant differences among plants (Fig. S6A). Thus, the TOP activity upsurge correlated with the activation of the local and systemic immunity.

Thiol homeostasis is essential for the activation of plant immunity (46). Although the cellular redox pathways involved in this process are not entirely defined, mutants with defects in the redox homeostasis exhibit immune-deficient phenotypes (47–50). Plants unable to express the chloroplast-localized NADPH-dependent thioredoxin reductase C (*NTRC*) over-accumulated oxidants and had high susceptibility to pathogenic *P. syringae* strains (51). We hypothesized that *NTRC*
might control TOP activity. To test this, we measured TOP activity in two mutants with altered NTRC expression—a knockout line (ntrc) and an overexpression line (NTRC-OE) (47, 48). In ntrc plants, we found equal TOP activity levels in PstAvrRpt2-challenged and buffer controls; notably, ntrc TOP activity reached values similar to those measured in pathogen-challenged Col-0 (Fig. 6C), an indication that in the absence of stress, NTRC acts as a negative regulator of TOP activity. No significant changes were found in the TOP activity in OE-NTRC extracts compared with Col-0 in control or pathogen-challenged plants (Fig. 6C).

We tested SAR activation in ntrc and OE-NTRC lines, as shown in Figure 6D. Measurements of PstDC3000 titer immediately after inoculation showed equal inoculum values among genotypes (Fig. S6A). However, at 48 hpi, PstDC3000 grew at similar levels in PstAvrRpt2-and buffer-challenged ntrc plants indicating that NTRC expression is required for SAR execution. On the other hand, overexpression of NTRC did not affect SAR, as shown by the wild-type level decrease in PstDC3000 multiplication in OE-NTRC plants (Fig. 6E). Considering the crucial role of SA-mediated signaling for the oxidative burst and SAR (52, 53), we tested the induction of canonical markers of SA-mediated signaling (PR1 and PAD4), SA synthesis (ICS1), and SAR (SAG13) in systemic leaves of ntrc. Compared with Col-0, the induction of these markers in ntrc was significantly suppressed (PR1 and SAG13) or abolished (ICS1 and PAD4) (Fig. 6F).

Augmented TOP activity during the plant immune response may result from TOP1 and TOP2 induction. Quantification of TOP1 and TOP2 mRNA levels in PstAvrRpt2-infiltrated and control leaves showed equal abundance in all samples for both transcripts (Fig. S6B); neither ntrc nor OE-NTRC lines showed changes in TOPs mRNA accumulation (Fig. S6C). An analysis of TOP1 and TOP2 expression patterns in published datasets (https://genevestigator.com/), in Col-0 and mutant lines with altered expression of immune components or defective redox homeostasis, during SAR or oxidative stress (H₂O₂), revealed no significant variation in TOPs expression levels (Fig. S6D).

We reasoned that TOP1 and TOP2 participate in the redox signaling activated following the PstAvrRpt2 localized challenge. We selected for this analysis the plant-specific transcription factors ZAT10 and WRKY33. Both are redox-regulated genes rapidly induced genes in response to ROS accumulation in locally stressed and unstressed systemic plants indicating that NTRC acts as a negative regulator of TOP activity. No significant changes were found in the TOP activity in OE-NTRC extracts compared with Col-0 in control or pathogen-challenged plants (Fig. 6C).

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Figure 7. Cleavage activity of plant TOPs on ROC1. A, HPLC chromatograms showing ROC1 peptide (ELYTDKTPRTAEN) elution peaks and retention times in the absence (control) or after incubation with TOP1, ΔSPTOP1, or TOP2. B, when ROC1p is treated with the inactive TOP mutants, only the full-length peptide (ELYTDKTPRTAEN, red, m/z = 769.38, +2 charge state) was detected in ESI-MS, indicating no cleavage. C, at a peptide:TOP reaction mixture ratio of 10:1 one cleavage site was detected for TOP1, ΔSPTOP1, and TOP2. The N-terminal product (ELYTDKTPR, blue, m/z = 561.79, +2 charge state) indicates this TOP cleavage site. D, at a peptide:TOP reaction mixture ratio of 1.5:1 two cleavage sites were detected for TOP1. The N-terminal product ELYTDKTPR indicates one TOP cleavage site. The N-terminal product ELYT indicates the second cleavage site. For ΔSPTOP1, the cleavage was indicated by the N-terminal product ELYT, while for TOP2, the cleavage was indicated by the N-terminal product ELYTDKTPR. All observed masses match with the theoretical peptide masses within 2 ppm mass error. The purity of synthesized peptide was determined to be ~60% through peak area analysis via LC-MS. E, diagram and table of all TOPs cleavage sites identified within ROC1p.
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Plant resistance to PstAvrRpt2 correlated with an upsurge in TOP activity (Fig. 6A). TOPs may be acting in the degradation pathway of a critical immune component of the PstAvrRpt2-triggered pathways. To identify possible plant-specific substrates of plant TOPs, we performed a BLAST search of the Arabidopsis proteome with a set of known substrates of THOP1 (EC:3.4.24.15) described in (58). We identified one protein, the rotamase cyclophilin 1 (ROC1/CYP18–3), in which the THOP1 (EC:3.4.24.15) cleavage (TA|EN) site was conserved. Notably, ROC1 is necessary for the self-cleavage of AvrRpt2 protease secreted into plant cells by P. syringae and subsequent processing of the immune regulator RIN4 (59). The activity of TOPs on ROC1 was tested using SEC and a ROC1 peptide (ROC1p) containing the THOP1 (EC:3.4.24.15) cleavage site (ELYTDKTPR|TAEN) (Fig. 7A and Table S1). In the control reaction without a TOP enzyme, ROC1p eluted as a single peak (Rt = 13.4 min, according to its molecular weight of 1430 Da). In the presence of TOP1 or ΔSP TOP1, an abundant ROC1p product eluted at Rt = 13.8 min, whereas in the absence of TOP2, a cleaved product eluted at Rt = 14.2 min. To verify that the SEC results peak from the enzymatic activity of TOPs on ROC1p, we generated two active site mutants. The first His residue in the active site motif HEXXH was replaced with Ala in both TOP1 and TOP2 to generate TOP1H571A and TOP2H483A (Fig. S7); a similar mutation in THOP1 (EC:3.4.24.15) and neurolysin abolished their enzymatic activity on natural substrates. In the presence of TOP1H571A or TOP2H483A, ROC1p eluted uncleaved (Fig. 7A).

Although all TOPs cleaved ROC1p, their cleavage site selection appeared to be dissimilar. We analyzed reaction mixtures containing TOPs at two different concentrations using electrospray ionization–mass spectrometry (ESI-MS) to determine cleavage site selectivity. In the presence of the inactive TOP1H571A or TOP2H483A, ROC1p eluted uncleaved (Fig. 7B). At 1:10 peptide:TOP ratio, the same cleavage site (ELYTDKTPR|TAEN) was identified for TOP1, ΔSP TOP1, and TOP2; for this cleavage site, the N-terminal product ELYTDKTPR was detected in all reaction mixtures and was most abundant in the ΔSP TOP1 reaction (Fig. 7, C and E). At the 1.5:1 peptide:TOP ratio, we identified a unique suite of cleavage products for each TOP isomorph. TOP1 produced two cleavage sites in ROC1p, including the site identified at 10:1 ratio (ELYTDKTPR|TAEN) detected by the presence of the abundant product ELYTDKTPR, and a second cleavage site (ELYTDKTPR|TAEN) detected by the presence of a comparatively smaller amount of the product ELYT (Fig. 7, D and E). At 1:5:1 with ΔSP TOP1, the ELYT product was detected, resulting from the ELYTDKTPR|TAEN cleavage; however, TOP2 cleaved ROC1p at the same site identified in the 1:10 reaction (ELYTDKTPR|TAEN), but more efficiently (Fig. 7, D and E). Thus, at high substrate concentrations, TOP isoforms cleave ROC1p at PR|TA with ΔSP TOP1 showing the highest efficiency. At low substrate concentration, PR|TA is a high-efficiency cleavage site of TOP1 and TOP2, whereas ΔSP TOP1 prefers YT|DK, which is also cleaved with lower efficiency by TOP1 (Fig. 7E).

Discussion

The biochemical characterization of the Arabidopsis TOPs revealed expected similarities and unexpected differences. We confirmed that although both TOPs are zinc-binding enzymes, similar to their mammalian counterparts, TOP2 binds multiple divalent cations with equal or higher affinity. This aspect merits further characterization as the potential functional role of metal selectivity in regulating catalytic activities of metalloproteases has been previously documented (60). An evaluation of TOPs redox-mediated self-interaction in E. coli found that both TOP1 and TOP2 monomers were stable under a broad range of redox conditions, and TOP1 was prone to forming higher-MW structures. Nevertheless, we interpreted the ex vivo assays’ results with prudence, given the nonlinear and concentration-dependent effects of redox treatments on growing E. coli in (61), where high oxidative stress caused increased GSSG accumulation. In contrast, lower oxidative stress had nonlinear and even opposite effects on the cells’ redox status.

Analyses of the purified TOPs complemented the ex vivo analysis by evaluating TOPs intrinsic sensitivity to redox agents. C52 and C611 were identified as critical residues by site-specific mutagenesis applied to investigate the structural basis of TOP1 dimerization. A mass spectrometric analysis to uncover the thiol status of TOP1 brought evidence that TOP1 forms disulfide-bonded homodimers; all cysteines in the signal peptide participated in disulfide bonds and C52 emerged as a participant in all detected disulfides. Measurements of Cys–Cys distances in the crystal structures of ΔSP TOP1 and TOP2 and our TOP1 model do not support the existence of intramolecular disulfides (the primary candidates being the conserved pair of “core” cysteines C548/460, C611/523 in TOP1/TOP2). However, our observations regarding the stability of Cys-to-Ala TOP1 mutants in E. coli suggest the possibility that certain redox conditions facilitate the formation of intramolecular disulfides in TOPs, as observed for...
other redox-sensitive plant proteins (25). Nevertheless, we cannot rule out the possibility that TOP2 dimerizes as well, as we observed DTT-red-sensitive dimers of a TOP2-Trx fusion in vitro and yeast cells (34). It remains to be seen if, in the plant cell, TOP2 is modified posttranslationally through interactions with more reactive redox sensors such as thioredoxins, or similar to THOP1 (EC:3.4.24.15) (62), undergoes other redox-mediated modifications such as S-glutathionylation. The formation of dimers and multimers in THOP1 (EC:3.4.24.15) was also attributed to intermolecular disulfides (62).

In plants, examples of proteins undergoing thiol–disulfide exchanges are limited; from the known examples, disulfides protect proteins against oxidative inactivation (63), inhibit (64), or have no observable effect (65). Our previous investigations of redox sensitivity of dimeric TOPs showed that the dimer was marginally less active than the monomer and that DTT inhibited their enzymatic activity to a similar extent (34). Thus, similar to TOPs in other eukaryotic systems, dimeric TOP1 maintains its activity; however, a detailed characterization of TOP1’s homotypic and heterotypic dimers may reveal differences. For TOP1, dimer formation may represent a regulatory mechanism in high oxidation environments through SA binding (41) and SA-mediated TOP1 dimerization (34); dimerization via transit peptide cysteines may also interfere with TOP1 transport into organelles by blocking its phosphorylation (66) or blocking interactions with translocons (67). Besides chloroplasts, TOP1 was detected in the proteomes of the cytosol (68), apoplast (69), and the plasma membrane following ETI activation by PstAvrRpt2 (70); thus, TOP1 may contribute to protein turnover in multiple subcellular compartments.

Measurements of subcellular thiol redox dynamics indicate that the plant cell cytosol is highly reducing (~320 mV); the estimated redox poised in mitochondria and light-exposed chloroplasts is even more negative (~380 mV) (71). Conversely, in the dark, when the strong reductive power of photosynthesis is missing, chloroplasts’ redox environment adjusts to lower potentials (i.e., up to ~240 mV) (72, 73). The redox poised of the cytosol and organelles undergoes dramatic shifts during an immune response (74), and the oxidative burst is a feature of both the local and systemic immunity (6, 75). We determined that oxidation increased TOPs activity; the estimated TOP1 midpoint redox potential of ~200 mV renders it active in more oxidative environments, suggesting a role during the pathogen-triggered oxidation waves. Its \( E_{m} \) value is within the range of other redox-active proteins such as thioredoxins and thioredoxin-regulated enzymes (42). Multiple cysteines in the transit peptide and peptidase domain of TOP1 contributed to its oxidative activation, suggesting that cystolic and organellar isoforms are both subject to redox regulation. On the other hand, the unique C\(^{405} \) was the most robust determinant of TOP2 oxidative activation. Enzyme activation by thiol oxidation plays a mechanistic role in many proteins. Activation of enzymes in the autophagy pathway during oxidative stress efficiently removed oxidized proteins (76); oxidation activated the cyclooxygenase COX (77) and the plant OXI1 necessary for redox-mediated signaling and pathogen resistance (78).

Consistent with the positive effect of oxidation on TOPs in vitro activity, we found that activation of local and systemic immunity correlated with enhanced TOP activity in the plant. Notably, TOP1 and TOP2 expression did not change significantly under various treatments and in mutant genotypes. Although we cannot account for possible variability in TOPs levels due to posttranscriptional events, the correlation between transcript and protein accumulation is high in Arabidopsis leaves (~70%, according to (79)). The study of NTRC mutants further supports the hypothesis that TOP activity is under redox regulation in the plant. The oxidative burst increases cellular oxidative potential, leading to TOP activity upsurge; the increased TOP activity of the nonstressed ntrc plants may be a consequence of the mutant’s increased ROS accumulation (51) and deficient thiol homeostasis (80). We postulate that NTRC, or a downstream component in the NTRC-mediated signaling, maintains TOPs in a reduced, low-activity state. Although our knowledge of the rich repertoire of plant thioredoxins is expanding, little is known about the range of oxidized residues they target, substrate selectivity, redox potentials, and specificity in redox-mediated pathways (81, 82). Notably, we found that ntrc plants cannot activate SA signaling and SAR, although it is unclear if these defects are a cause or consequence of defective redox regulation of TOPs. In the top1top2 knockout line, induction of ROS marker genes was deregulated, with an overall increase in local and systemic gene activity. These results point to an adverse effect of TOPs on ROS-responsive gene induction. The elevated redox marker expression in top1top2 is consistent with its augmented hypersensitive response and cell death (25, 28). These results support a role for TOP1 and TOP2 to SAR regulation through the modulation of redox-sensitive pathways.

To begin disentangling TOPs role in immune redox signaling, to this end, we searched for immune-associated TOP substrates. TOPs cleaved the key immune component, ROC1, at sites different from the THOP1’s, and site selection was dependent on the substrate’s concentration. ROC1 plays an essential role in plant–pathogen recognition by promoting the P. syringae AvrRpt2 protease maturation; this effector protein is secreted in plant cells during infection and activates ETI following its recognition by the RPS2 immune receptor (59). Although classified as a cytosolic rotamase (83), ROC1 was identified in the plastid stroma (84), mitochondria (85), plasma membrane (86, 87), and extracellular proteome (69). The importance of TOP1 and TOP2 proteolysis of ROC1 for the plant immune response remains to be explored.

We rationalized in vitro and in vivo observations to signify that changes in the plant cell’s redox environment modify sensitive TOPs thiol and affect their activity. Reducing conditions prevalent in nonstressed plants during the light cycle would keep TOPs activity at low levels; oxidative stress triggered by pathogen infection and in nonphotosynthesizing chloroplasts would augment TOPs activity via thiol oxidation. Increased TOP activity in systemic tissues may contribute to immune priming, whereby specific cellular defense responses are operationally prepared for augmented expression upon pathogen attack but with yet little-understood molecular mechanisms (88). Further studies of TOP substrates and
downstream signaling pathways may reveal a more widespread role of redox-mediated proteolysis on plant immunity.

**Experimental procedures**

**Molecular modeling and biochemical analyses**

Chimera (89, 90) was used to visualize and analyze the known molecular structures of \( \text{ASS}^+ \)TOP1 and TOP2 and to obtain a model of the full-length TOP1 and calculate the Cys–Cys distances within the 3D structures. For the thermal denaturation of TOPs, time-resolved intensity decay for tryptophan was measured using a spectrophuorometric instrument Dls DM 45 at 280 nm excitation and 300–500 nm for the fluorescence emission.

**Cloning and site-directed mutagenesis**

The cDNA sequences encoding \( A. \text{thaliana} \) TOP1 (AT5G65620) and TOP2 (AT5G10540) were cloned into pET-28a (Agilent) in a translational frame with 6xHis N-terminal tags. Cysteine mutagenesis was performed by site-directed mutagenesis, using the wild-type cDNAs, gene-specific primers, and Phusion High-Fidelity DNA Polymerase (New England Biolabs). The PCR products were separated on agarose gels (0.8%), purified using the QIAquick gel extraction Kit (Qiagen), and cloned into pET-28a using NheI and SalI restriction enzymes (NEB) and T4 DNA ligase (NEB). The plasmids were used for the expression of TOPs in \( E. \text{coli} \). Additional details are described in Supplemental Methods.

**Protein expression and purification**

Plasmids containing the native and mutant TOPs were transformed into competent \( E. \text{coli} \) NiCo21 (DE3) cells (NEB). \( E. \text{coli} \) cultures were grown with shaking at 37 °C in LB Broth media in the presence of 50 mg/L kanamycin. At OD_{600} around 0.5–0.6, the cultures were induced with Isopropyl 2-D-1-thiogalactopyranoside (IPTG) at 100 mg/L and collected after 4 h. Pellets were stored at –80 °C for at least 1 day, then thawed on ice, and lysed in B-PER complete bacterial protein extraction reagent (ThermoFisher) before processing. The lysed cells were centrifuged at 4000 rpm for 30 min at 4 °C. The supernatants were concentrated in an Amicon stirred cell (50 ml) with Ultrafiltration discs (PLGC, Ultracel regenerated cellulose, 30 kDa) and then purified via batch HisPur Ni-NTA Resin (Thermo Fisher Scientific) following the manufacturer’s protocol. Before analysis, proteins were dialyzed overnight with 50 mM Tris-buffer pH 7.0 using membrane tubes (6–8 kDa) at 4 °C overnight and concentrated before 30 min-long treatments with a 10-fold molar excess of oxidative and reductive agents as described in (39). The reducing agents used were L-Glutathione reduced (GSH) and 1, 4-Dithiothreitol (DTTred); the oxidizing agents were L-Glutathione oxidized (GSSG), trans-4, 5-dihydroxy-1, 2-dithiane (DTTox), and hydrogen peroxide (H₂O₂). To obtain a range of redox potentials, GSH, GSSG, DTTred, and DTTox were mixed in defined ratios (39). Additional details are described in Supplemental Methods. We analyzed the dependence of TOP1 activity on the redox potential using DTTred/DTTox and GSH/GSSG. \( E_m \) values were estimated by fitting oxidation ratio \((R)\) calculated with \( R = \frac{F_{ox}}{F_{ox} + F_{red}} \) \( (F_{ox} \) and \( F_{red} \) are the fraction of enzyme at oxidized and reduced conditions, respectively) to calculated potentials of redox couples using Nernst equation, \( E_h = E_m + (RT/ nF)\ln(\frac{[F_{ox}]}{[F_{red}]})) \), with RT/F = 25.693 mV and n-number of electrons exchanged in the redox reaction. The enzyme activity reduction ratio was estimated from fluorescence measurements of enzyme activity over the substrate (initial reaction rates). The fitting equation \((Y = 1/(1 + \exp(X - E_m) n/ 25.693))\) was implemented using GraphPad PRISM v8 as a nonlinear least square fitting method. Calculation of TOPs \( E_m \) value is based on the equilibrium midpoint potential values of DTTred/DTTox redox couple (~327 mV) and GSH/GSSG redox couple (~240 mV) at pH 7.0.

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**PAGE electrophoresis and immunoblotting**

Bacterial cultures expressing wild-type and mutant TOPs were grown in Luria–Bertani (LB) medium containing kanamycin (50 mg/L) to OD_{600} = 0.6 induced 4 h with 100 mg/L IPTG. \( E. \text{coli} \) cells were collected by centrifugation, and pellets were lysed using B-PER complete bacterial protein extraction reagent. The Bradford assay determined protein concentration before equal amounts of proteins were loaded into PAGE gels and run using a BIO-RAD electrophoresis system. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes using the semidyrid transfer method at 20 V for 40 min, blocked in 5% nonfat milk, probed with the primary antibody (6x-His Tag Monoclonal Antibody) (1:500), and then the secondary antibody Goat anti-Mouse IgG (H + L) HRP conjugated (1:5000) (Thermo Fisher) at room temperature. Membranes were incubated with ECL substrate (Bio-Rad), and the bands were visualized using a Bio-Rad imaging system. For native PAGE, purified TOPs were mixed with Native PAGE 4× sample loading buffer to a final concentration 1x, loaded in a native PAGE, and ran at 200 V using a Bio-Rad system. PageBlue stain (Thermo Scientific) was used to visualize protein bands as described by the manufacturer. For immunoblotting, proteins were transferred to PVDF membranes and processed as described.
**Enzymatic activity assays**

TOP enzymatic activity was measured by using an Olis DM 45 spectrophotometric instrument. Purified TOPs were concentrated to 10 μg and incubated in 500 μl of 50 mM Tris-buffer pH 7.0, supplemented with 100 mM NaCl, with 8 μM of the fluorogenic substrate Mca-PLGPK(DNP)-OH (Mca–7-methoxycoumarin-4-acetyl–DNP–2,4-dinitrophenol) (Enzo LifeSciences) or the ROC1 peptide (AB Clonal). The cleavage of the fluorogenic substrate was monitored by \( \lambda_{\text{ex}} \) at 330 nm and \( \lambda_{\text{em}} \) at 400 nm, and TOP activity measurements in plant extracts were done as described in (28). We measured the initial reaction rate (μM s\(^{-1}\)) of TOPs activity from the in vitro reactions (first 100 s time points) using methods implemented in a recently developed online tool (93). We used the logarithmic approximation method (94), except for controls, where we used a linear method (95), to minimize the standard error for the time interval of measurements. TOP1 and TOP2 progression curves of reaction product (cleaved substrate concentration) were measured at a range of redox potentials. In tissue lysates, TOP activity was estimated by calculating the normalized total reaction product (integrated using the area under curve tool in GraphPad PRISM v8 [https://www.graphpad.com/scientific-software/prism/]) per time unit. The product reaction concentration was normalized per microgram of total protein.

**Plant material and growth conditions**

*Arabidopsis thaliana* ecotype Columbia (Col-0), NTRC-OE, and ntrc mutant seeds were sterilized as described in (96), grown on MS media for 10 days, then transferred to a controlled growth chamber with a 12 h light and 12 h dark setup, 100 μmol m\(^{-2}\) s\(^{-1}\) photon flux density, and relative humidity of 60 to 65%. Day and night temperatures were set to 23 °C and 21 °C, respectively.

**Pathogen assays**

*P. syringae pv. tomato (Pst)* strain DC3000c AvrRpt2 was cultivated at 28 °C in King’s B medium. Pathogen assays were performed as described in (97). Briefly, overnight log-phase cultures were diluted to final optical densities (OD\(_{600}\)) for leaf inoculations. For ETI, PstAvrRpt2 was suspended in MgCl\(_2\) (1 × 10\(^5\) CFU/ml) before infiltration in the leaves of mature plants. For SAR, one lower (1°) leaf was infiltrated with a PstAvrRpt2 suspension of 1 × 10\(^7\) CFU/ml; 48 h postprimary infiltration, upper leaves were harvested for the determination of TOPs expression and activity or infiltrated with PstDC3000 (1 × 10\(^7\) CFU/ml). Experiments were performed with 4-week-old plants with a uniform appearance.

**Quantitative PCR analysis**

Total RNA was extracted using the RNA extraction kit (Sigma Aldrich). One microgram of total RNA was used for cDNA synthesis with the iScript gDNA Clear cDNA Synthesis kit (Bio-Rad). Transcript levels were measured using SYBR Green technology and EvaGreen qPCR mix plus (Solis BioDyne). Data were analyzed using the ΔΔCT method (98) and the endogenous control ubiquitin.

**High-performance liquid chromatography (HPLC)**

His-tagged TOP1 and TOP2 purified from *E. coli* and dialyzed were treated with redox agents with gentle mixing for 30 min at RT. The redox reagents have a strong absorbance at 280 nm; therefore, after treatments and before HPLC, proteins were subjected to second purification and redialyzed in 50 mM Tris buffer at pH 7.0 supplemented with 100 mM NaCl to avoid protein aggregation. The HPLC was performed using an XBridge BEH200A SEC 3.5 μm 7.8 × 300 mm column connected to an Agilent 1100 series HPLC system. The column was equilibrated with HPLC water for 30 min, followed by 50 mM Sodium phosphate buffer containing 150 mM NaCl and 0.02% NaN\(_2\) at pH 7.0, for 30 min. The isocratic mode was used to run the sample for 30 min with 50 mM sodium phosphate buffer with 150 mM NaCl and 0.02% NaN\(_2\) at a flow rate of 0.88 μl/min. Chromatograms were recorded and processed with the Agilent HPLC software B.04.03.

**In-gel digestion and LC-MS/MS analysis**

The native PAGE gel was excised manually around the masses for the TOP1 monomer and dimer for downstream processing. Gel slices were destained three times with 300 μl of 50 mM ammonium bicarbonate/50% ACN solution before in-gel trypsin digestion (5 μl of 0.5 μg/μl trypsin in 50 mM acetic acid) was performed in 50 mM ammonium bicarbonate overnight at RT. Following trypsin digestion, 10 mM calcium chloride was added, and in-gel chymotrypsin digestion (5 μl of 0.5 μg/μl chymotrypsin in 1 mM hydrochloric acid) was performed in the same solution for 4 h at RT. Peptides were extracted first with 30 μl of 1% formic acid/2% ACN, followed by 30 μl of 60% ACN. Peptides were dried by vacuum centrifugation, resuspended in 1 ml of 0.1% TFA, and desalted by reversed-phase solid-phase extraction as described above before LC-MS/MS. Samples were resuspended in 5% acetonitrile/0.1% TFA and analyzed using an Acquity UPLC M-Class System (Waters) coupled to a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific). Mobile phase A consisted of water with 0.1% formic acid (Thermo Fisher Scientific), and mobile phase B was acetonitrile with 0.1% formic acid. Injections were made to a Symmetry C18 trap column (100 Å, 5 μm, 180 μm × 20 mm; Waters) with a flow rate of 5 μl/min for 3 min using 99% A and 1% B. Peptides were then separated on an HSS T3 C18 column (100 Å, 1.8 μm, 75 μm × 250 mm; Waters) using a linear gradient of increasing mobile phase B at a flow rate of 300 nL/min. Mobile phase B was held at 5% for 1 min, then increased from 5% to 50% in 30 min before ramping to 85% in 2 min, where it was held for 3 min before returning to 5% in 1 min and re-equilibrating for 23 min. The mass spectrometer was operated in positive polarity, and the Nanospray Flex source had spray voltage floating at 2.1 kV, the capillary temperature at 320 °C, and funnel RF level at 40. MS survey scans were collected with a scan range of 350–2000 m/z at a resolving power of 120,000 and an AGC target of 3 × 106 with a maximum injection time of 50 ms. A top 20 data-dependent acquisition was used where HCD fragmentation of precursor ions having +2 to +7 charge state was performed using a normalized collision energy setting of 28. MS/MS scans were performed at a resolving power
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of 30,000 and an AGC target of 1 x 105 with a maximum injection time of 100 ms. Dynamic exclusion for precursor m/z was set to a 10 s window.

Proteomics database searching

Acquired spectral files (*.raw) were converted into combined peak lists (*.mgf) using MSConvertGUI (version 3.0.18130-20e9d0c75b5) (99) for peptide sequence determination by Mascot (Matrix Science, version 2.5.1). Database searching was performed against the A. thaliana UniProt database (https://www.uniprot.org/proteomes/UP000006548, 39,359 canonical entries, accessed April 24, 2020) and E. coli UniProt database (https://www.uniprot.org/proteomes/UP000002032, 4156 canonical entries, accessed December 4, 2020) with sequences for common laboratory contaminants (https://www.thegpm.org/cRAP/, 116 entries, accessed April 24, 2020) appended. Target-decoy searches of MS/MS data used a trypsin (specificity: K/R that are not before P)/chymotrypsin (specificity: F/Y/W/L that are not before P) protease specificity with the possibility of two missed cleavages, peptide/fragment mass tolerances of 15 ppm/0.2 Da, and variable modifications of N-terminus acetylation and methionine oxidation. Significant peptide identifications above the identity or homology threshold were adjusted to less than 1% peptide FDR using the embedded Percolator algorithm (100). The threshold for a peptide–spectra match was Percolator-adjusted Mascot Peptide Score >13.

In vitro enzyme assay, solid-phase extraction, and ESI-MS analysis

The custom synthesized ROC1 peptide ELYTDKPRTAEN (AB Clonal) sequence was synthesized in 500 μl 50 mM Tris, pH 7.5. To initiate the enzyme assay, TOP1, ASPTOP1, TOP2, TOPH71D, or TOPA1483D was added at a peptide:TOP ratio of 1.5:1 and 10:1. The reaction mixture was incubated at 23 °C for 30 min. Before ESI-MS analysis, desalting was performed using 50 μg/1.0 ml Sep-Pak C18 cartridges (Waters) held in an SPE manifold (Phenomenex) at a flow rate of 1 drop/s. Resin was first pre-eluted using 1 ml of 80% acetonitrile/0.1% TFA before equilibration with 1 ml of 0.1% TFA. Samples were acidified to pH 3 using 10% TFA and loaded onto the cartridges in two passes, and then washed using 1 ml of 0.1% TFA. Peptides were eluted using 1 ml of 80% acetonitrile/0.1% TFA and concentrated by vacuum centrifugation to dryness. Samples were resuspended in 100 μl of 50% methanol/0.1% formic acid. Peptides were directly infused via ESI on a Thermo Q Exactive HF-X Hybrid mass spectrometer for intact mass analysis. Samples were injected at a flow rate of 10 μl/min, and full MS scans were analyzed in the Orbitrap. The mass spectrometer was operated at a resolving power of 120,000, positive polarity, spray voltage of 3 kV, with 150–2000 m/z range, and collecting 100 scans per sample for averaging.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository and can be accessed with the dataset identifier PXD024059 and 10.6019/PXD024059. Username: reviewer_pxd024059@ebi.ac.uk; password: zhjMfoSh.

Supporting information—This article contains supporting information (39).

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Abbreviations—The abbreviations used are: ESI-MS, electrospray ionization–mass spectrometry; ETI, effectors-triggered immunity; GSH, glutathione; NTRC, NADPH-dependent thioredoxin reductase C; OOP, organellar oligopeptidase; ROS, reactive oxygen species; SAR, systemic acquired response; SEC, size-exclusion chromatography; TOP, thimet oligopeptidase.

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