

COI1 phosphorylation status drives redox state-mediated thermotolerance in plants

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Abstract

High temperature increasingly threatens crop growth and yield under climate change. Plants rely on phytohormone signalling to adapt to heat stress, yet the underlying mechanisms remain poorly understood. One of the plant hormone receptors, the jasmonate receptor CORONATINE-INSENSITIVE 1 (COI1), plays an essential role in conferring plant thermotolerance. However, to date, the molecular mechanisms by which COI1 mediates plant high temperature tolerance remain enigmatic. Here, we characterise moderately high temperature-regulated differential phosphorylation of TaCOI1-1D, one of the wheat COI1 homeologous proteins. We show that TaCOI1-1D is a functional component of the Skp1–Cullin–F-box COI1 (SCF^{COI1}) complex and that its heat-regulated phosphosite, Ser16, is conserved with Thr16 in Arabidopsis COI1. Mutating this site enhances the COI1–ARABIDOPSIS SKP1 HOMOLOGUE 1 (ASK1) interaction in both wheat and Arabidopsis. We further demonstrate that Arabidopsis COI1 interacts with the nucleocytoplasmic monothiol glutaredoxin glutaredoxin S17 (GRXS17). The enhanced COI1–ASK1 binding stabilizes COI1 and promotes the release of GRXS17, allowing the formation of GRXS17 oligomers, a possibly essential state for AtGRXS17's function in regulating H₂O₂ scavenging and enhancing thermotolerance in Arabidopsis. Our findings reveal a mechanism in which a moderately high temperature-induced modification of the JA receptor simultaneously stabilizes the receptor and orchestrates redox-dependent thermotolerance.

INTRODUCTION

High temperature, frequently associated with climate change, negatively impacts plant growth and development and often leads to losses in crop seed yield and quality^{1–3}. To cope with temperatures above those for optimal growth and to guarantee survival under high temperature stress, plants have evolved overlapping but distinct cellular responses, including basal thermotolerance, short- and long-term acquired thermotolerance, and thermotolerance to moderately high temperatures^{4–8}. These cellular responses include heat stress transcription factors and heat shock proteins, which protect cellular proteins and maintain proteostasis^{5,9}. Additionally, high levels of reactive oxygen species (ROS) accumulate during heat stresses, leading to cellular damage¹⁰. Thus, plants must control the production and scavenging of ROS to protect against stress damage^{10–12}. High temperature responses also involve several phytohormone pathways^{4,13–18}, such as jasmonate (JA). Prolonged exposure to warm temperature induces JA catabolism, which reduces the levels of bioactive jasmonates and is essential for thermomorphogenesis¹⁹. In contrast, the role of JA in temperature responses appears to depend on the intensity and duration of the temperature increase. Under acute heat stress, bioactive jasmonates accumulate and contribute to the activation of heat stress responses^{17,20–23}. Thus, JA metabolism and signalling are differentially regulated depending on the temperature regime and the physiological response involved. Importantly, moderately high temperature promotes the stability of CORONATINE INSENSITIVE1 (COI1), the canonical receptor for the bioactive JA conjugate JA-Ile^{19,24}; and a *coi1* loss-of-function mutant displays compromised thermotolerance in Arabidopsis²⁰.

COI1 is the F-box component of an Skp1–Cullin–F-box (SCF) ubiquitin ligase complex and perceives bioactive JA-Ile, leading to proteasome-dependent degradation of JASMONATE ZIM-DOMAIN (JAZ) transcriptional repressors to de-repress various transcription factors, such as MYCs, inducing a substantial transcriptional reprogramming^{25–30}. COI1 protein stability is maintained by ARABIDOPSIS SKP1 HOMOLOGUE 1 (ASK1) and ASK2, and COI1 is rapidly degraded via an SCF^{COI1}-independent 26S proteasome pathway in the absence of ASK1/2³¹. In addition to ASK1/2, the SGT1b–HSP70–HSP90 chaperone complex also helps to stabilize COI1^{24,32}.

In this study, we discovered that the phosphorylation status of wheat (*Triticum aestivum*) TaCOI1-1D – one of the functional COI1 paralogs in wheat – is regulated by moderately high temperature. The Ser16 phosphosite in TaCOI1-1D and the corresponding Thr16 phosphosite in Arabidopsis COI1 modulate the COI1-ASK1 interaction. In Arabidopsis, an enhanced COI1-ASK1 interaction maintains COI1 protein stability upon moderately high temperature stress, and promotes the release of a previously unknown COI1 interactor, glutaredoxin S17 (GRXS17). GRXS17 in turn modulates hydrogen peroxide (H₂O₂) levels during the recovery following moderately high temperature stress, therefore conferring plant thermotolerance.

Results and discussion

Moderately high temperature-mediated phosphorylation of wheat COI1 proteins

Thermotolerance to (moderately) high temperatures (referred to as thermotolerance below) enables sustained growth and development upon (prolonged) exposure to moderately elevated temperatures, as described before^{6–8}. Following up on the differences in growth between different wheat varieties³³ (**Supplementary Fig. 1a-f**), we observed that the wheat varieties ‘Fielder’ and ‘Cadenza’ showed better thermotolerance than the wheat variety ‘Reeves’ (Fig. 1a-b, **Supplementary Fig. 1g**). Since JA signalling plays an important role during thermomorphogenesis¹⁹ and high temperature stress response^{17,20–23}, we explored phosphorylation of the jasmonate signalling-associated proteins COI1 and JAZ in our previously published phosphoproteome data sets of wheat exposed to heat^{34,35}, and we only observed changes in the – possibly regulatory – phosphorylation of wheat COI1 proteins (Fig. 1c-d, **Supplementary Fig. 2 and Supplementary Table S1**). We observed a significant change in the abundance of a TraesCS1D02G278400 (TaCOI1-1D) phosphopeptide containing Serine 16 in ‘Fielder’ and ‘Cadenza’ when comparing 24°C and 34°C, but we could not detect the associated phosphopeptide in ‘Reeves’^{34,35} (Fig. 1c). Notably, this was the only phosphopeptide detected across all COI1 homeologs. This indicates a correlation between the absence or the moderately high temperature-mediated downregulation of this phosphopeptide and thermotolerance or response to moderately high temperature, respectively. In contrast, a similar phosphopeptide from TraesCS1A02G279100 (TaCOI1-1A) or TraesCS1B02G288100 (TaCOI1-1B) showed comparable levels when comparing 24°C and 34°C ‘Cadenza’ and ‘Reeves’, while we

could not detect this phosphopeptide in 'Fielder' (Fig. 1d). These results suggest that phosphorylation status of TaCOI1-1D, and likely not of TaCOI1-1A or TaCOI1-1B, is possibly involved in moderately high temperature response and thermotolerance in wheat.

Wheat COI1-1D is a functional component in SCF complexes

Given its differential phosphorylation, we focused on TaCOI1-1D for our subsequent analyses and we first validated whether TaCOI1-1D is part of a functional SCF^{COI1} complex³⁶. In *Arabidopsis thaliana*, COI1 proteins are known to interact with other SCF^{COI1} components through ASK1^{30,31}. Therefore, we first randomly selected four wheat ASK1-like proteins from all wheat ASK1-like proteins (**Supplementary Fig. 3**)³⁷ to test the interaction with TaCOI1-1D. Our yeast-two hybrid (Y2H) results showed that these four TaASK1 proteins interacted with TaCOI1-1D (**Supplementary Fig. 4a**). For one wheat ASK1 orthologue (TaASK1-7D), which is the closest orthologue of rice ASK1 (OSK1)³⁸, we further confirmed the interaction with TaCOI1-1D through co-immunoprecipitation (co-IP) assays following transient expression in *Nicotiana benthamiana* leaves (**Supplementary Fig. 4b**). In addition to ASK1, JAZ proteins also interact with COI1 in a coronatine- or JA-Ile-dependent manner^{27,36,39}. We randomly selected four wheat JAZ-like proteins from all wheat JAZ-like proteins (**Supplementary Fig. 5**)⁴⁰. Our Y2H analyses showed that TraesCS5B02G203400 (TaJAZ9-5B), TraesCS2A02G169300 (TaJAZ1-2A), TraesCS2B02G195600 (TaJAZ1-2B) and TraesCS2D02G176800 (TaJAZ1-2D) interacted with TaCOI1-1D in a coronatine-dependent way³⁹ (**Supplementary Fig. 5, Supplementary Fig. 6a**). Since the rice OsCOI1b, a close ortholog of TaCOI1-1D (**Supplementary Fig. 2**), has been shown to interact with and regulate degradation of OsJAZ9, we focused on TaJAZ9-5B, which is the closest ortholog of OsJAZ9³⁸, for further analyses.

The F-box domain is essential for F-box proteins to assemble the SCF E3 ligase complex and subsequently regulate polyubiquitination and subsequent degradation of substrates (e.g., JAZ proteins) in a 26S proteasome-dependent way^{31,36,38,39}. When transiently co-expressing TaJAZ9-5B together with TaCOI1-1D in *N. benthamiana* leaves, we detected very little to no TaJAZ9-5B protein (**Supplementary Fig. 6b-d**). Furthermore, the TaJAZ9-5B protein remained stable when co-expressed with a TaCOI1-1D protein variant with the F-box domain deleted (TaCOI1-1D^{ΔFbox}) (**Supplementary Fig. 6b-d**). In addition, two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins are essential for the coronatine- and JA-Ile-dependent AtCOI1-AtJAZs interaction^{29,41}. An AtJAZ9 variant with R223A and K224A amino acid substitutions in the Jas motif abolishes binding to AtCOI1, and thus prevents AtJAZ9 protein degradation in response to JA treatment⁴¹. We identified the corresponding positively charged residues (Arg335 and Lys336) in TaJAZ9-5B (**Supplementary Fig. 7**), and generated a TaJAZ9-5B variant with R335A and K336A substitutions (mTaJAZ9-5B). Indeed, mTaJAZ9-5B could not bind to TaCOI1-1D in a Y2H assay (**Supplementary Fig. 6a**), and mTaJAZ9-5B was more stable compared to TaJAZ9-5B when co-expressed with TaCOI1-1D (**Supplementary Fig. 6b**).

Furthermore, GFP:TaCOI1-1D specifically localizes to the nucleus in Arabidopsis when expressed under the Arabidopsis native *COI1* promoter (*pAtCOI1::GFP:TaCOI1-1D*) (**Supplementary Fig. 8**), and expressing *pAtCOI1::GFP:TaCOI1-1D* in the Arabidopsis *coi1-1* could restore sensitivity to MeJA-mediated growth inhibition (**Supplementary Fig. 9a-b**) and male fertility^{36,42} (**Supplementary Fig. 9c**). These observations support that TaCOI1-1D plays a similar molecular role as AtCOI1.

Finally, we identified two independent Cadenza wheat TILLING lines with a premature stop codon (Q367* and W540*) in the coding sequence of *TaCOI1-1D* (**Supplementary Fig. 9d**). Both TaCOI1-1D Cadenza wheat TILLING lines showed longer second leaf and sheath length at 14°C and 19°C, compared to the corresponding wild type plants (**Supplementary Fig. 9e-l**). However, a warm temperature (29°C)-mediated increase of second leaf and sheath length was lacking in both TaCOI1-1D Cadenza wheat TILLING lines, compared to the corresponding wild type plants (**Supplementary Fig. 9i-l**). Taken together, the phenotyping results from two independent TaCOI1-1D TILLING lines supported a growth repressing role for TaCOI1-1D, which is in line with our previous observations for Arabidopsis *coi1-21*¹⁹.

All the evidence together supports that *TaCOI1-1D* (*TraesCS1D02G278400*) encodes a functional COI1 protein and is part of SCF^{COI1} complexes involved in JA responses.

Unphosphorylated COI1 displays both enhanced interaction with ASK1 and an increased COI1 protein stability

Next, we explored the functional importance of TaCOI1-1D phosphorylation at Ser16. As the identified Ser16 phosphosite is close to the F-box domain (**Supplementary Fig. 9d**), which is essential for COI1 interacting with ASK1^{30,31}, we investigated if replacing Ser16 by Ala16 affected the interaction between COI1 and ASK1. Indeed, probing the interaction between the phospho-mutant TaCOI1-1D^{S16A} and TaASK1-7D in a Y2H assay showed a stronger interaction compared to the interaction between wild-type TaCOI1-1D and TaASK1-7D (Fig. 2a-b). In addition, a co-immunoprecipitation assay also showed that TaASK1-7D bound much stronger to TaCOI1-1D^{S16A} than to TaCOI1-1D (Fig. 2c- d). This supports the idea that dephosphorylation of COI1 at Ser16 contributes to its interaction with ASK1.

To gain a deeper mechanistic insight in COI1 phosphorylation and its impact, we used *Arabidopsis thaliana* as a model system for further analyses. A closer analysis of the TaCOI1-1D and AtCOI1 sequences showed that Ser16 of TaCOI1-1D aligned with Thr16 (and possibly Ser12) in AtCOI1 (Fig. 2e). To rule out that Ser12 in AtCOI1 might have a similar function as Ser16 in TaCOI1-1D, we explored the impact of exchanging either Ser12 to Ala12 or Thr16 to Ala16. Using Y2H and co-immunoprecipitation assays, we revealed that AtCOI1^{T16A} enhanced the interaction with AtASK1, while an AtCOI1^{S12A} variant had no obvious impact (Fig. 2f-i, **Supplementary Fig. 10a-b**). This corroborates the conserved importance of COI1 phosphorylation at position 16 for interaction with ASK1. In addition, we attempted to identify phosphorylation sites in AtCOI1 using immunoprecipitation-mass spectrometry (IP-MS) on transgenic Arabidopsis seedlings overexpressing GFP-tagged AtCOI1. Unfortunately, while we could

detect an increase in abundance of the non-phosphorylated COI1 peptide containing T16 at 34°C compared to 21°C (**Supplementary Fig. 10c, Supplementary Table S2**), no phosphopeptides corresponding to AtCOI1 could be detected at 34°C and 21°C.

Association of COI1 with ASK1 in SCF complexes is essential for maintaining COI1 protein stability^{30,31}. Thus, we wondered if the enhanced COI1-ASK1 interaction affected COI1 protein stability. For this, Arabidopsis COI1:GFP or COI1^{T16A}:GFP driven by the Arabidopsis native *COI1* promoter were expressed in the *coi1-1* mutant background (**Supplementary Fig. 11a-b**). Both *pAtCOI1::AtCOI1:GFP coi1-1* (AtCOI1 hereafter) and *pAtCOI1::AtCOI1^{T16A}:GFP coi1-1* (AtCOI1^{T16A} hereafter) lines displayed the same COI1 nuclear localization (**Supplementary Fig. 12**). We did not detect an obvious difference in COI1 protein abundance in AtCOI1 and AtCOI1^{T16A} seedlings exposed to 21°C (Fig. 2, j and k, **Supplementary Fig. 11b**). However, moderately high temperature (34°C) resulted in a higher increase of AtCOI1^{T16A} compared to AtCOI1 upon a 5-hour 34°C treatment (Fig. 2j-k, **Supplementary Fig. 11c-d**). Since *AtCOI1* steady state transcript levels are not markedly affected in the AtCOI1 and AtCOI1^{T16A} seedlings exposed to 21°C or 34°C (**Supplementary Fig. 13a**), this supports that the changes in AtCOI1 protein level are not regulated at the transcriptional level. These results indicate that unphosphorylated COI1 enhances the COI1-ASK1 interaction to promote and maintain COI1 protein levels during and after moderately high temperature stress, respectively.

An AtCOI1 variant improves thermotolerance to moderately high temperatures

To explore the significance of COI1 dephosphorylation, we assessed warm temperature-mediated hypocotyl elongation and thermotolerance at moderately high temperature. First, we showed that the expression of transcriptional markers for high temperature response^{4,5,43,44} is similar at 21°C and equally highly induced at 34°C in both AtCOI1 and AtCOI1^{T16A} lines (**Supplementary Fig. 13b**). This indicates that the overall temperature perception and immediate response are not affected. Second, we did not observe an obvious difference in thermomorphogenesis, as assessed by warm temperature-induced hypocotyl elongation, for *AtCOI1* and *AtCOI1^{T16A}*-expressing *coi1-1* seedlings at 21°C and 28°C (**Supplementary Fig. 14**). This further supports our previous study that warm temperature-triggered hypocotyl elongation is largely caused by the reduction in bio-active JA-Ile levels¹⁹, and does not strongly depend on COI1 protein activity and/or COI1 abundance. Third, JA signalling contributes to Arabidopsis thermotolerance and (moderately) high temperature induces the accumulation of JA and JA-Ile²⁰. We therefore hypothesized that higher COI1 protein levels or stabilized COI1 protein in the *AtCOI1^{T16A}*-expressing seedlings possibly play a role in enhancing thermotolerance. To validate this hypothesis, we assessed recovery from moderately high temperature. One-week old *AtCOI1* and *AtCOI1^{T16A}*-expressing seedlings grown at 21°C were exposed to moderately high temperature (37°C) for 4 days and then transferred back to 21°C for 10 days (Fig. 3a). AtCOI1^{T16A} plants tolerated the moderately high temperature stress much better, as reflected in dramatically increased recovery,

compared to the AtCOI1 plants (Fig. 3b). Together, our observations indicate that increased COI1 protein levels or stability correlate with increased recovery capacity from exposure to moderately high temperatures.

An AtCOI1 variant improves recovery from moderately high temperature through modulating ROS homeostasis

To elucidate the mechanisms underlying COI1-mediated thermotolerance, we performed IP-MS on a *GFP-AtCOI1*-expressing line exposed to 21°C and 34°C (**Supplementary Table S3**). GO enrichment analysis on the *GFP-AtCOI1* IP-MS dataset revealed that SCF complex-related GO categories were enriched at both 21°C and 34°C, supporting the temperature-independent activity of the SCF^{COI1} complex (**Supplementary Fig. 15 and Supplementary Tables S3 to S6**). However, redox-related GO categories were more enriched at 21°C compared to 34°C (**Supplementary Fig. 15 and Supplementary Tables S3 to S6**). Since modulation of ROS levels leads to stress tolerance^{46,47}, JA represses the ROS wave⁴⁸, and ROS functions as a signalling molecule to mediate JA signalling in plant⁴⁹, we explored if there is a link between ROS and AtCOI1^{T16A}-mediated thermotolerance. For this, we analysed ROS levels, such as superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), in *AtCOI1* and *AtCOI1*^{T16A}-expressing seedlings before, during and after exposure to moderately high temperature. Both 3,3'-diaminobenzidine (DAB) staining and H_2O_2 quantification assays revealed that *AtCOI1*^{T16A}-expressing seedlings could remove H_2O_2 during the recovery from moderately high temperature more efficiently compared to *AtCOI1*-expressing seedlings (Fig. 3c-d). However, there was no obvious difference in superoxide radical ($O_2^{\cdot-}$) levels between *AtCOI1* and *AtCOI1*^{T16A}-expressing seedlings at each condition (**Supplementary Fig. 16**). These results indicate that during the recovery stage after exposure to moderately high temperature AtCOI1^{T16A} modulates H_2O_2 levels, which might explain the observed difference in thermotolerance between *AtCOI1* and *AtCOI1*^{T16A}-expressing seedlings.

AtASK1–COI1–GRXS17 module regulates H_2O_2 levels during moderately high temperature stress recovery

To further investigate how AtCOI1 mediates H_2O_2 scavenging, we looked into the *GFP-AtCOI1* IP-MS dataset for ROS-related proteins that preferentially interacted with COI1 at high temperature (**Supplementary Tables S3 to S5**), where we detected glutaredoxin S17 (GRXS17). GRXS17 has a cytosolic role in iron-sulphur cluster biogenesis^{50,51} and has both foldase and redox-dependent holdase activities at higher temperatures and under oxidative conditions⁵². In addition, re-analysis of a tandem affinity purification coupled with mass spectrometry using GRXS17 as bait⁵³ also identified COI1, but not ASK1, as an interactor (**Supplementary Table S7**), finding COI1 and GRXS17 in two independent MS datasets (ref.⁵³ and our data) strongly suggested a robust and biologically relevant interaction. And we further validated *GFP-AtCOI1* and *AtGRXS17-RFP* interaction through co-immunoprecipitation (co-IP)

following transient expression in *N. benthamiana* (Fig. 4a). Furthermore, AtGRXS17-RFP co-localizes with GFP-AtCOI1 in the cytosol and the nucleus (**Supplementary Fig. 17**). Since Arabidopsis GRXS17 contributes to thermotolerance and modulates H₂O₂ levels upon higher temperatures^{52,54}, we further focused on this candidate.

We subsequently showed that *grxs17-1* mutant plants exhibited reduced efficiency in H₂O₂ scavenging compared to wild-type plants during recovery from moderately high temperature stress (Fig. 4b-c). Consequently, *grxs17-1* and *grxs17-4* mutant plants exhibited impaired recovery following moderately high temperature stress (**Supplementary Fig. 18a-b**), which is consistent with earlier findings^{52,54}. In addition, AtGRXS17 complements the moderately high temperature-sensitive phenotype of the *grxs17-1* mutant (**Supplementary Fig. 18b**). To investigate if AtGRXS17 is required for H₂O₂ removal and thermotolerance of *AtCOI1*^{T16A}-expressing lines, we crossed *AtCOI1* and *AtCOI1*^{T16A}-expressing lines with the *grxs17-1* mutant. Interestingly, 3,3'-diaminobenzidine (DAB) staining and H₂O₂ quantification assays revealed that *AtCOI1*^{T16A}-expressing seedlings in the *grxs17-1* background failed to efficiently remove H₂O₂ during the recovery from moderately high temperature exposure compared to *AtCOI1*^{T16A}-expressing seedlings (Fig. 4d-e). Furthermore, *AtCOI1*^{T16A}-expressing seedlings could not recover from moderately high temperature stress in the *grxs17-1* mutant background (Fig. 4f). These results indicate that AtGRXS17 is required for H₂O₂ removal and thermotolerance in *AtCOI1*^{T16A}-expressing lines.

Next, as COI1 is a component of an SCF ubiquitin ligase complex, and GRXS17 is a known target for E3 ligases⁵³, we tested if AtCOI1 affects AtGRXS17 protein abundance. To bypass the male sterility associated with *coi1-1*⁵⁵, the alternative *coi1-2* allele was used⁵⁶. We observed that, while AtGRXS17 protein level increased slightly at 37°C, there was no significant difference in AtGRXS17 protein level in the wild-type (Col-0) and *coi1-2* mutant lines at either 21°C or 37°C (**Supplementary Fig. 19a-b**). Moreover, slightly increased levels of AtCOI1 and increased levels of *AtCOI1*^{T16A} did not lead to lower levels of GRXS17 (**Supplementary Fig. 19c-e**). Those results suggest that AtGRXS17 is likely not recruited by the SCF^{COI1} ligase complex for ubiquitination and degradation. We further questioned if AtGRXS17 affects AtCOI1 protein abundance. Both *grxs17-1* mutant and 35S::*AtGRXS17-GFP* transgenic seedlings displayed a comparable accumulation of AtCOI1 protein upon different duration exposure of higher temperature compared to Col-0 (**Supplementary Fig. 18c-d**), which suggests that GRXS17 does not affect COI1 protein abundance.

Since *AtCOI1*^{T16A} exhibits a stronger interaction with AtASK1 than wild-type *AtCOI1*, we investigated whether AtASK1 influences the interaction between *AtCOI1* and AtGRXS17. For this, GFP-*AtCOI1* or GFP-*AtCOI1*^{T16A}, 3×HA-AtASK1, and AtGRXS17-RFP were co-expressed in *N. benthamiana* leaves and subjected to co-immunoprecipitation assays. Interestingly, GFP-*AtCOI1*^{T16A} co-precipitated less AtGRXS17-RFP and more 3×HA-AtASK1 than GFP-*AtCOI1* (Fig. 5a-c). However, in the absence of 3×HA-AtASK1 in the co-IP assay, GFP-*AtCOI1* and GFP-*AtCOI1*^{T16A} co-precipitated a comparable amount of AtGRXS17-RFP (**Supplementary Fig. 20**). These results suggest that AtASK1 competes with AtGRXS17

to bind to AtCOI1. To further support this conclusion, we mutated tryptophan 44 of AtCOI1 to alanine (AtCOI1^{W44A}) to substantially weaken its interaction with AtASK1⁵⁷ (Fig. 5c, **Supplementary Fig. S21**). Indeed, co-expression in *N. benthamiana* leaves followed by co-IP assays revealed a significantly stronger interaction between AtGRXS17-RFP and GFP-AtCOI1^{W44A} compared to GFP-AtCOI1 (Fig. 5a-c).

Because AtCOI1 protein purification structurally requires co-expression and purification with AtASK1 to maintain solubility and stability, setting up a conventional *in vitro* biochemical competitive binding assay with isolated components was technically not feasible. To circumvent this purification bottleneck and evaluate the competitive mechanism with structural resolution, we utilized an *in silico* modelling approach combining AlphaFold 3 and AlphaBridge interface analyses (**Supplementary Fig. 22**). In the wild-type ternary complex, AlphaBridge mapped a balanced network of shared interface contacts where AtCOI1 (Chain C) successfully establishes contact ribbons with both AtASK1 (Chain B) and AtGRXS17 (Chain A) (**Supplementary Fig. 22a**). In contrast, modelling of the AtCOI1^{T16A} mutant complex revealed a pronounced structural shift (**Supplementary Fig. 22b**). In line with our co-IP observations, the T16A substitution led to an enrichment and tightening of the contact ribbons bridging AtCOI1 (Chain C) to AtASK1 (Chain B). Concurrently, this enhanced AtCOI1-AtASK1 interface visually outcompeted and diminished the interaction boundary with AtGRXS17 (Chain A), demonstrating how the T16A mutation shifts the equilibrium toward an AtASK1-bound state at the expense of the AtGRXS17 assembly. These results demonstrate that AtASK1 competitively inhibits the interaction between AtCOI1 and AtGRXS17, with enhanced AtASK1 binding to AtCOI1 leading to reduced association with AtGRXS17.

AtGRXS17 exists in different forms, including as a monomer or oligomer, with distinct functions⁵². Oxidative stress in combination with heat stress induces the formation of disulphide bonds between conserved active-site cysteines in the corresponding thioredoxin domains of GRXS17⁵². The disulphide bonds lead to the formation of GRXS17 oligomers and thus activates its holdase activity to protect plants from stresses^{52,58}. We hypothesized that a stronger COI1-ASK1 interaction releases GRXS17, which forms oligomers that act as a holdase to positively regulate H₂O₂ scavenging and regulate thermotolerance. Since H₂O₂ in combination with moderately high temperature (37°C) induces AtGRXS17 oligomerization⁵², GFP-AtCOI1 or GFP-AtCOI1^{T16A}, 3×HA-AtASK1, and AtGRXS17-RFP fusion proteins were co-expressed in *N. benthamiana* leaves in the presence of H₂O₂ and subjected to non-reducing native-PAGE and western blot analyses. In addition, the AtGRXS17^{C33/179/309/416S} variant (referred to as AtGRXS17^{4C/S}), which is fully unable to oligomerize⁵², was included as a control (Fig. 5d and e, **Supplementary Fig. 23**). We observed that AtGRXS17-RFP oligomer intensity was higher in samples co-expressing GFP-AtCOI1^{T16A} compared to samples with GFP-AtCOI1, while AtGRXS17^{4C/S} could not oligomerize (Fig. 5d and e, **Supplementary Fig. 23**). We questioned whether the enhanced AtGRXS17-RFP oligomer intensity was caused by reduced association with GFP-AtCOI1^{T16A} compared with GFP-AtCOI1. Consistent with the reducing SDS-PAGE result (Fig. 5a-b), AtGRXS17-RFP co-precipitated less GFP-AtCOI1 complex in samples co-expressing GFP-AtCOI1^{T16A} compared to samples with GFP-AtCOI1 (**Supplementary Fig. 23**). Together, these results indicate that enhanced AtASK1

association with AtCOI1^{T16A} reduces the AtCOI1–AtGRXS17 interaction, releasing AtGRXS17 and thereby facilitating AtGRXS17 oligomerization under oxidative and moderately high temperature conditions.

CONCLUSION

Taken together, our results support a model in which COI1 regulates redox homeostasis through dynamic, phosphorylation-dependent switching between its interactions with GRXS17 and ASK1 in Arabidopsis (Fig. 5f). Under control conditions, we hypothesize that phosphorylated COI1 preferentially associates with GRXS17, limiting its interaction with ASK1. This is supported by reduced phosphorylation at the conserved position 16 in wheat COI1 from thermotolerant wheat varieties upon exposure to moderately high temperature, and our biochemical analyses further indicate that the corresponding Thr16 residue in Arabidopsis COI1 is important for its interaction with GRXS17 and ASK1. Although we were unable to directly detect the phosphorylated Thr16-containing peptide of Arabidopsis COI1 by IP-MS, the increased abundance of the non-phosphorylated Thr16-containing peptide at elevated temperature is consistent with, but does not by itself establish, a temperature-dependent change in Thr16 phosphorylation. Thus, the available evidence supports a conserved functional role of the position-16 residue in regulating the COI1-ASK1-GRXS17 module, while the extent to which its phosphorylation status is directly regulated by temperature may differ or remains to be established in Arabidopsis.

Within this model, reduced phosphorylation at Thr16 is proposed to enhance COI1 association with ASK1, thereby releasing GRXS17 to form oligomers, a state that may be important for GRXS17 function in H₂O₂ scavenging and thermotolerance (Fig. 5f). Consistent with this model, the unphosphorylated and more stable COI1 protein in COI1^{T16A}-expressing Arabidopsis plants interacts less with GRXS17, which is associated with improved tolerance and recovery from moderately high temperature, potentially through maintenance of JA signalling and modulation of ROS homeostasis (Fig. 5f). Likely, this regulatory pathway acts in parallel with other redox-sensitive systems. For instance, our model provides an additional mechanism to the H₂O₂-mediated sulfenylation of GRF8, which similarly serves as a redox-based checkpoint to modulate JA signalling in Arabidopsis⁴⁹. Precisely how these distinct regulatory elements are interconnected and coordinated in the context of temperature acclimation remains an open question for future research. Overall, our findings indicate that phosphorylation-dependent regulation of COI1 stability and protein-protein interactions contributes to the functional coordination of the COI1-ASK1-GRXS17 module during heat stress and suggest that this regulatory module may represent a potential target for improving thermotolerance in crops.

MATERIALS AND METHODS

Plant material and growth conditions

A. thaliana plants used in this study were in the Col-0 reference accession genetic background or in *coi1-1*⁵⁵. The Arabidopsis *coi1-2*⁵⁶, *grxs17-1* (SALK_021301)^{51,52}, *grxs17-4* (*grxs17* Cas9-3)⁵⁹, *grxs17-1* +

GRXS17 (*p35S::AtGRXS17-GFP grxs17-1*)⁵² and *p35S::AtGRXS17-GFP*⁵² were previously characterized. For the *coi1* alleles, the *coi1-1* mutant is a complete loss-of-function null allele that causes absolute male sterility⁴². Therefore, *coi1-1* must be maintained as heterozygote, making it highly effective for generating and screening complementation lines via floral dip, but less practical for obtaining a large, uniform population of homozygous mutant plants for physiological or biochemical experiments. To overcome this limitation for some experiments, we used the leaky mutant allele *coi1-2*⁶⁰. The *coi1-2* allele retains severe jasmonate insensitivity, but exhibits partial male fertility, allowing us to reliably harvest and work with large batches of uniform, homozygous mutant seeds. For protein extraction, seeds were surface sterilized and vertically grown on square plates with medium containing ½ Murashige-Skoog basal salt (½ MS) (Duchefa), 1% agar, and 1% sucrose. Seeds were first stratified for 2 days at 4 °C and then transferred to a growth chamber at 21 °C, with continuous light (70–100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 50% relative humidity for germination. After germination, plates were transferred to long-day conditions of 16 h light/8 h dark at 21 °C (75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation supplied by light emitting diode (LED) lights). For moderately high temperature treatment, plates were transferred to 21 °C (control) and 34 °C for 5 hours. The whole seedlings were collected for protein extraction in the afternoon of the 7th day after germination. For protein extraction to detect Arabidopsis COI1 protein level using anti-COI1 antibody, 6- or 14-day-old seedlings were grown at 21 °C in long-day condition (16h light/8h dark) (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation supplied by light emitting diode (LED) lights) on ½ MS without sucrose solid medium, then 6- or 14-day-old seedlings were transferred to an incubator set at 35 °C in long-day conditions for 0, 2, 6 and 24 hours. For protein extraction to detect Arabidopsis GRXS17 protein level, 5- or 7-day-old seedlings were grown at 21 °C in long-day condition (16h light/8h dark) (75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation supplied by light emitting diode (LED) lights) on ½ MS with 1% agar solid medium, then 5- or 7-day-old seedlings were transferred to an incubator set at 21 °C (control) or 37 °C in long-day conditions for 1 or 2 days. For MeJA treatment, seeds that germinated uniformly were transferred to a medium as mentioned above supplemented with DMSO as mock or 30 μM MeJA (Sigma-Aldrich, CAS: 39924-52-2), then plates were transferred to long-day conditions of 16 h light/8 h dark at 21 °C (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation supplied by cool-white, fluorescent tungsten tubes, Osram). Plates were scanned 7 days after MeJA treatment. Arabidopsis plants used for fertility observation were grown in plastic pots containing a jiffy peat pellet in a growth chamber at 21 °C, long-day conditions of 16 h light/8 h dark at 21 °C (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation supplied by cool-white, fluorescent tungsten tubes, Osram). The wheat seeds used in this study were from hexaploid wheat cultivars: ‘Felder’, ‘Cadenza’, and ‘Reeves’. The seeds were placed on wet paper, enclosed in plastic wrap, and kept at 4 °C for 3–4 days, and then transferred to room temperature in the dark for 1 day for germination. Seeds that germinated uniformly were selected and grown in plastic pots containing soil at the temperatures of interest under 16 h light/8 h dark (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation, supplied by cool-white, fluorescent tungsten tubes, Osram) and 65–75% air humidity. Phenotyping pictures were taken with a Canon digital camera. Measurements were done using Fiji/ImageJ (<https://imagej.net/Fiji>). *Nicotiana benthamiana* L. plants were grown in soil [3 parts of Grow Mix Multipro (Terafertil, Argentina) with 1 part of vermiculite]

with a 16-h photoperiod ($150 \mu\text{E m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation) at 25 °C and 60% relative humidity in a greenhouse. For transient expression of proteins of interest, leaves from 4-week-old *N. benthamiana* were infiltrated with *Agrobacterium tumefaciens* strain C58 carrying the constructs of interest together with *P19* (a gene silencing suppressor) as described⁶¹. Infiltration solution (10 mM MgCl_2 , 10 mM MES pH 5.6, 100 μM acetosyringone) was used as control. Infiltrated leaves were harvested 72 h post-infiltration and stored at -80 °C. For the hydrogen peroxide (H_2O_2) and moderately high temperature treatments, *Nicotiana benthamiana* leaf material from 2 independent leaves was collected 72 hours post-infiltration (hpi). Protein expression was initially verified using a Leica SP8 confocal laser scanning microscope (Leica Microsystems, Germany) according to the detailed settings described below. Following confirmation of robust protein expression, the leaves were infiltrated with 5 mM H_2O_2 ^{46,62,63} prepared in infiltration solution. The plants were immediately transferred to a 37 °C incubator under continuous light for 5 hours. Following incubation, the treated leaves were harvested and stored at -80 °C.

Wheat TILLING lines

The wheat COI1 ortholog ID in the D genome (TraesCS1D02G278400) was searched on the EnsemblPlants website (plants.ensembl.org/Triticum_aestivum) to find TILLING mutations (www.wheat-training.com). The heterozygous wheat *coi1* TILLING lines^{64,65}, Cadenza0877 (Q367*) and Cadenza1333 (W540*), were ordered directly through the UK Germplasm Resource Unit website (www.seedstor.ac.uk). The seeds were sown into soil in the growth chamber and cultivated at 21 °C day (16 h)/18 °C night (8 h), under light levels of $700 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 70% relative humidity, to confirm the plants with and without the mutation of interest. For this, the youngest leaf of individual plants at 1-2 weeks post-germination was collected for genotyping. Sample DNA was extracted by using a protocol for DNA extraction in 96-well plates (www.wheat-training.com) adapted from Pallota M.A et al (2003)^{66,67} and PCR was performed using primers listed in **Supplementary Table S8**. The 25 μl PCR reaction was performed according to the manufacturer's instructions, and the size-confirmed PCR product was sequenced. Subsequently, the point mutation was confirmed by sequencing using primers listed in **Supplementary Table S8**. Homozygous mutant plants were backcrossed with wild type (Cadenza0000) to reduce the load of background mutations, followed by self-pollination. For phenotyping, seeds from plants with and without the mutation of interest were placed on wet paper enclosed in plastic wrap and kept at 4 °C for 3–4 days, and then transferred to room temperature for germination. Seeds that germinated uniformly were selected and grown in plastic pots containing soil at 14 °C under 16 h light/8 h dark ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation, supplied by cool-white, fluorescent tungsten tubes, Osram), and 65–75% air humidity. Phenotyping pictures seedlings after a 2-week temperature treatment were taken with a Canon digital camera.

Gene cloning, site-directed mutagenesis, and plasmid construction

PCR was performed using iProof™ High-Fidelity DNA Polymerase according to the manufacturer's instructions (Bio-Rad). The coding sequences of Arabidopsis *COI1* and *ASK1*, which were amplified from cDNA made from total mRNA extract of Arabidopsis Col-0 seedlings, and *GRXS17*, which was described previously⁵³, were cloned into the pDONR221 or pDONR207 gateway entry vectors (ThermoFisher SCIENTIFIC), respectively. The CDS of wheat *TraesCS1D02G278400* (*COI1-1D*), *COI1-1D*^{ΔFbox}, *TraesCS6D02G001600* (*ASK1-6D*), *TraesCS7D02G016900* (*ASK1-7D*), *TraesCS2D02G560500* (*ASK1-2D*), *TraesCS4A02G366400* (*ASK1-4A*), *TraesCS5B02G203400* (*JAZ9-5B*), *TraesCS2A02G169300* (*JAZ1-2A*), *TraesCS2B02G195600* (*JAZ1-2B*), *TraesCS2D02G176800* (*JAZ1-2D*) and *Os08g0428400* (*OsJAZ9*) were synthesized and domesticated for BsaI using the TWIST Bioscience (twistbioscience.com). All the synthesized coding sequences were assembled into BsaI-linearised pEN-L1-AG-L2 by NEBuilder HiFi DNA Assembly kit. Specific mutations were introduced into the *AtCOI1* and *TaCOI1-1D* coding sequences directly through the Quick-Change Site Directed mutagenesis kit (Agilent Technologies). The pDONR221 (*AtCOI1*) and pEN-L1-AG-L2 (*TaCOI1-1D* CDS) were used as templates for PCR with specific primers containing the mutation site. Then, the DNA template plasmid was digested with DpnI for 1 h before being transformed into *DH5α* *E. coli* cells. In addition, to generate the mutation of *AtGRXS17* and *TaJAZ9-5B*, the C33/179/309/416S mutations on *AtGRXS17* CDS and R335/K336A mutations on *TaJAZ9-5B* were generated by overlapping polymerase chain reaction, assembled into BsaI-linearised pEN-L1-AG-L2 by NEBuilder HiFi DNA Assembly kit. A fragment of 1055 base pairs upstream of *COI1* CDS (called *COI1* promoter or *pAtCOI1*) was amplified by PCR from genomic DNA extracted from Col-0 seedlings and cloned into the pDONR P4-P1r plasmid gateway entry vector (ThermoFisher SCIENTIFIC). All entry vector plasmids were checked by PCR and sequenced using appropriate primers (**Supplementary Table S8**).

For Y2H assays, the wild-type and mutated Arabidopsis and wheat *COI1* coding sequences in the entry vector were recombined into pGILDAgate using LR ClonaseII (ThermoFisher SCIENTIFIC) to generate C-terminal fusions to the LexA DNA binding domain. The *AtASK1* and *TaASK1* orthologues, *AtJAZ9*, *TaJAZ1-2A/B/D*, and *TaJAZ9-5B* or *TaJAZ9-5B*^{R335A/K336A}, *OsJAZ9* coding sequences were recombined into pB42ADgate to generate C-terminal fusions to the B42 transcriptional activation domain.

For transient expression in *N. benthamiana*, the wild-type and mutated Arabidopsis and wheat *COI1* coding sequences in the entry vector were recombined into pB7WGF2, and all JAZ coding sequences were recombined into pB7WGR2 using LR ClonaseII (ThermoFisher SCIENTIFIC) to generate N-terminal fusions to the GFP and RFP under the control of *CaMV* 35S promoter. Wild-type and mutated *AtGRXS17* coding sequences without stop codons were recombined into pK7RWG2 using LR Clonase II (ThermoFisher SCIENTIFIC) to generate C-terminal fusions to the RFP under the control of *CaMV* 35S promoter. In addition, 3×HA, *AtASK1*, and *TaASK1-7D* coding sequences in the entry vector were recombined into pB7m24GW2 to make N-terminal fusions to HA under the control of *CaMV* 35S promoter.

For plant transformation, the *AtCOI1* sequence without a stop codon was cloned into pDONR221. Next, the 1055 bp native promoter of *AtCOI1* (*pAtCOI1*), *AtCOI1*, and *AtCOI1*^{T16A}, *GFP* in the entry vector were

recombined into the pBb7m34GW vector using LR Clonase II Plus. For Golden Gate cloning, the native promoter of *AtCOI1* and *TaCOI1-1D* CDS were cloned into pGGA000 and pGGC000, respectively ⁶⁸. To generate *pAtCOI1::GFP-TaCOI1-1D* construct, the respective fragments were assembled into the destination vector pGGB-AG ⁶⁹. In addition, *p35S::GFP-AtCOI1*, *p35S::GFP-AtCOI1^{T16A}*, and *p35S::GFP-AtCOI1^{W44A}* which were generated for transient expression in *N. benthamiana* above were also used for stable transgenic plants. Confirmed constructs were introduced into *Agrobacterium tumefaciens* C58C1 using the freeze-thaw method ⁷⁰, and plant transformation was performed using the floral dip method as previously described ⁷¹. All transgenic plants contain a BastaR resistance marker.

Yeast two-hybrid and β -Galactosidase assays

The yeast EGY48 strain carrying the *p8Op:LacZ* reporter plasmid ⁷² was co-transformed with the appropriate combinations of fusion constructs carrying the B42 activation domain (AD) or the LexA DNA-binding domain (BD) using the polyethylene glycol (PEG)/lithium acetate method according to the instructions provided with the Matchmaker LexA Two-Hybrid System (#K1609-1, Clontech). Colonies were selected on minimal synthetic defined (SD) plates (Clontech) supplemented with the -uracil (U)/-tryptophan (T)/-histidine (H) amino acid drop-out solution (Clontech). At least three yeast colonies were cultured overnight in liquid SD-UTH medium. For Y2H on plates, 10- μ l and 10-fold dilution of the overnight cultures were spotted onto SD galactose/raffinose-UTH plates with 80 μ g/ml X-gal (Sigma-Aldrich). For COI1-JAZ protein interactions, 10 μ M coronatine must also be added to SD galactose/raffinose-UTH plates containing 80 μ g/ml X-gal (Sigma-Aldrich). Blue colour indicated protein–protein interactions after 3–5 days at 30 °C incubation. β -Galactosidase activities were measured according to the Yeast Protocols Handbook (Clontech). For liquid culture assay, galactosidase activity was analysed using o-nitrophenyl- β -D-galactopyranoside (ONPG; Sigma Cat No. N-1127) as a substrate (β -galactosidase units = $1000 \times OD_{420} / [t \times V \times OD_{600}]$, where t is elapsed time [in min] of incubation, $V = 0.1$ mL $\times$ concentration factor, and $OD_{600} = A_{600}$ of 1mL of culture). At least three independent experiments were performed for each interaction pair.

Arabidopsis transformation and screening

p35S::GFP-AtCOI1^{WT/T16A} constructs were transformed into wild-type Arabidopsis Col-0. *pAtCOI1::COI1^{WT/T16A}-GFP* and *pAtCOI1::GFP-TaCOI1-1D* constructs were transformed into the Arabidopsis *coi1-1* heterozygous mutant ⁵⁵. Because homozygous *coi1-1* plants are male-sterile, heterozygous plants were identified through genotyping as previously described ⁵⁵. Briefly, a 1446-bp fragment containing part of *COI1* or *coi1-1* was PCR-amplified by the primers (**Supplementary Table S8**). XcmI (#R0533S, NEW ENGLAND Biolabs) cleaved the 1446-bp fragment from the WT DNA, but not from *coi1-1* in which the XcmI recognition site CCA-9N-TGG was altered by the *coi1-1^{G1401A}* mutation to CCA-9N-TGA ⁵⁵. Then, heterozygous plants were used for *A. tumefaciens*-mediated Arabidopsis transformation. Half-strength Murashige and Skoog (MS) medium with 50 μ g/mL Basta was used to select transgenic T1 seedlings containing *p35S::GFP-AtCOI1^{WT/T16A}*, *pAtCOI1::AtCOI1^{WT/T16A}-GFP*, and

pAtCOI1::GFP-TaCOI1-1D transgene. Basta-resistant seedlings were transplanted, and genotyping was performed to identify transgenic plants with the homozygous *coi1-1* background. Further screening of T2 or T3 plants was performed for the homozygous transgene. Primers used for *coi1-1* are listed in **Supplementary Table S8**.

Protein extraction, co-immunoprecipitation, and western blot

For protein extraction using transgenic plants driven by the native *AtCOI1* promoter, 10 seedlings of each line were frozen in liquid nitrogen, grinded by Retsch MM400, and homogenized in 100 µl ice-cold homogenization buffer (400 mM sucrose, 25 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1% Trion X-100, 0.1 mM DTT, 0.1 mM β-mercaptoethanol, 0.5 mM PMSF and a Roche Complete protease inhibitor; 1 tablet/10 ml). The tubes were incubated on ice for 10 min, and then the extracted protein samples were sonicated using sonicator baths (10 seconds on, 10 seconds off, 20 cycles). After sonification, tubes were centrifuged for 15 min at 20,817 × g at 4 °C, and the desired amount of supernatant was transferred to a new 1.5 ml tube and mixed with Laemmli sample buffer (Bio-Rad, USA) and NuPAGE™ Sample Reducing Agent (Thermo Scientific, USA) and incubated at 95°C for 5 min. For protein extraction for co-immunoprecipitation and SDS-PAGE, in brief, the plant material was suspended with cold reducing extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40 (v/v), 10% glycerol (v/v), 10 mM EDTA, 1 mM DTT, 1 mM sodium molybdate, 1 mM NaF and 1 mini tablet of Roche Complete protease inhibitor per 10 mL buffer). For protein extraction for co-immunoprecipitation and native-PAGE, the plant material was suspended with cold non-reducing extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40 (v/v), 10% glycerol (v/v), 10 mM EDTA, 1 mM sodium molybdate, 1 mM NaF and 1 mini tablet of Roche Complete protease inhibitor per 10 mL buffer). The samples were centrifuged for 30 min at 20,817 g at 4°C, then filtered with Miracloth (MERCK, Germany) to remove debris. Depending on the experiment, each sample was mixed with 20 µl of either magnetic GFP or RFP-Trap beads (Chromotek, USA) or Anti-HA magnetic beads (Thermo Scientific, USA) and incubated at 4°C for 1-1.5 h with rotation. The supernatant was removed from the beads using a magnetic rack. Beads were subsequently washed three times, each with 1 ml wash buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl). Samples were mixed with 1× NuPAGE LDS sample buffer and NuPAGE™ sample reducing agent (Thermo Scientific, USA) and incubated at 95°C for 5 min. In addition, samples for native-PAGE were mixed with native sample buffer (Bio-Rad, USA) and incubated at 70°C for 5 min. Proteins were separated on 4–20% Mini-PROTEAN® TGX Stain-Free™ Gels (Bio-Rad Laboratories, Inc., USA) for SDS-PAGE or Native-PAGE, then transferred onto a Trans-Blot® Turbo™ Mini PVDF pack (Bio-Rad Laboratories, Inc., USA). Membranes were blocked for 1 h at room temperature in 4.5% Difco™ Skim Milk (BD Life Sciences, USA). After blocking, membranes were washed 4-5 times using PBS-T (PBS with 0.1% TWEEN® 20 (Sigma-Aldrich, USA). Depending on the experiment, the membrane containing the proteins was incubated with anti-GFP-HRP (Chromotek, USA) (1:10000), anti-RFP (mouse) (Chromotek, USA) (1:2000), and anti-mouse secondary antibody at 1:10000 dilution (Cytiva, USA), anti-HA-HRP (Chromotek, USA) (1:10000), anti-GRXS17 (rabbit) (1:5000) was described previously⁵³, and anti-rabbit secondary antibody at 1:10000 dilution (GE Healthcare), anti-α-Tubulin (mouse) (Sigma, USA) (1:5000). anti-COI1(rabbit) (1:3500). The proteins were

detected by using a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Inc., USA). Strain-free gel imaging or Coomassie staining was used for the sample loading control. Depending on the experiment, membranes were stripped using stripping buffer (1:1–1.5 ml 10% SDS: 1.5 ml 100 mM Glycine mix) for 90 s. Membranes were washed 4–5 times using dH₂O, followed by 3–4 times washing with PBS-T. Membranes were blocked for 1 h at room temperature and washed 4–5 times with PBS-T as described above for the other antibody incubation.

Protein quantification

Quantification for relative protein intensity was performed by measuring protein bands using Fiji (ImageJ). For each lane, target bands and their respective loading controls were selected using the image analysis tools to determine their integrated density. Local background intensity was accounted for by manually measuring a blank area adjacent to the bands and subtracting it from the raw band values. For standard western blots, target protein expression was normalized to its loading control: either the intensity of the Rubisco large subunit (RbcL) band visualized on the stain-free gel/ coomassie blue staining, or the immunodetected anti-tubulin band. For co-immunoprecipitation (co-IP) assays, the intensity of the target protein in the IP fraction was normalized to its respective expression levels in the magnetic GFP-Trap or anti-HA magnetic beads-enriched protein fraction. A single sample lane was designated as the reference control. The relative quantity for each experimental band was calculated by dividing its normalized, background-adjusted intensity value by the normalized, background-adjusted intensity of the reference band. Consequently, the value of the reference band is set to 1.0. A value greater than 1.0 indicates that the protein levels exceed the reference intensity, while a value less than 1.0 indicates it is lower than the reference intensity.

Gene expression analyses

Samples from 7-days-old Arabidopsis seedlings were harvested. Three or four biological replicates were performed for each tested gene. Total RNA from Arabidopsis seedlings was extracted and purified with the RNeasy Mini Kit (Qiagen). DNA digestion was done on columns with RNase-free DNase I (Promega). The Superscript cDNA Synthesis Kit (Quantabio) was used for cDNA synthesis from 1 µg of RNA. qRT-PCR was performed on a LightCycler 480 (Roche Diagnostics) in 384-well plates with LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer's instructions. *ACTIN-RELATED PROTEIN 7* (*ARP7*) and the *TRANSLATIONAL ELONGATION FACTOR ALPHA* (*EF1α*) genes were used as Arabidopsis internal reference genes. All primers are listed in **Supplementary Table S8**. For the quantification of *AtCOI1* transcript abundance in *pAtCOI1::AtCOI1^{WT/T16A}-GFP coi1-1* transgene, qAtCOI1 forward and reverse primers amplify a portion of coding sequence that is before the premature stop codon (*coi1-1* mutant background) (**Supplementary Fig. 11a**). Analysis of relative gene expression data was performed using the $2^{-\Delta\Delta CT}$ method.

Confocal microscopy

Five-day-old *pAtCOI1::AtCOI1-GFP* or *pAtCOI1::AtCOI1^{T16A}-GFP coi1-1* seedlings or *N. benthamiana* leaf disks were collected and imaged on a Leica SP8 confocal laser scanning microscope (Leica Microsystems, Germany). Detectors settings used for one experiment were optimized to avoid saturation and to maximize resolution and kept unchanged throughout the experiment. GFP was excited using the 488 nm line (Leica SP8). GFP fluorescence was collected from 493 to 532 nm (using the AOBS of the SP8). RFP was excited using a 561-nm laser diode (SP8). RFP fluorescence was collected from 590 to 680 nm (using the AOBS of the SP8). Gating technology was applied to remove autofluorescence. Images were taken using the ×40 objective.

Protein extraction and sample preparation for immunoprecipitation followed by LC-MS/MS The procedure has been described previously⁷³ with some modifications. For the high temperature-treated *A. thaliana* seedlings, seeds were surface sterilized and vertically grown on square plates with medium containing ½ Murashige-Skoog basal salt (½ MS) (Duchefa), 1% agar, and 1% sucrose. Seeds were first stratified for 2 days at 4 °C and then transferred to a growth chamber at 21 °C, with continuous light (70-100 µmol m⁻² s⁻¹) at 50% relative humidity for germination. After germination, plates were transferred to long-day conditions of 16 h light/8 h dark at 21 °C (75 µmol m⁻² s⁻¹ photosynthetically active radiation supplied by light emitting diode (LED) lights) to grow 10 days. For moderately high temperature treatment, plates were transferred to 21 °C (control) and 34 °C for 1 hours. Whole seedlings were collected for protein extraction in the afternoon of the 10th day after germination. Each biological replicate contains around 2 gram fresh weight. Three biological replicates were harvested and ground to a fine powder using liquid nitrogen. Proteins were extracted in extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 0.5% IGEPAL CA-630, supplemented with protease and phosphatase inhibitors), incubated on ice for 30 min with gentle rotation, and centrifuged at 4 °C. Supernatants were filtered and incubated with GFP-Trap Magnetic Agarose beads pre-equilibrated in diluted extraction buffer. After 2 h incubation at 4 °C with rotation, beads were washed three times with wash buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl) and once with 50 mM TEAB (pH 8.0). The on-bead digestion was performed with 0.5 µg trypsin in 50 mM TEAB. The peptides in the supernatant were alkylated on cysteine residues using 10 mM TCEP and 30 mM iodoacetamide. After reduction with 5 mM DTT, the peptide solution was treated with an additional 0.5 µg of trypsin and digested overnight. Digestion was stopped by adding TFA to a final concentration of 1%. Desalting was performed by OMIX C18 pipette tips according to the manufacturer's guidelines, and elution in 60% ACN, 40% water, 0.1% TFA. The samples were vacuum dried and analysed by LC-MS/MS.

LC-MS/MS analysis, database searching and data analysis

The procedure has been described previously⁷³ with some modifications. Samples were analysed by LC-MS/MS using an Ultimate 3000 RSLC nano LC (Thermo Fisher Scientific) in-line connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific). The samples were loaded on an analytical column (made in-house, 25 cm column, 1.9 µm beads, 75 µm internal diameter, Dr. Maisch, Ammerbuch-Entringen, Germany) with solvent 0.1% TFA in water. The samples were separated by solvent A (0.1%

formic acid in water) and solvent B (0.1% formic acid in water/ACN, 20/80 [v/v]) with a linear gradient from solvent A/solvent B, 98/2 (v/v) to solvent A/solvent B, 45/55 (v/v) over 2.5 hour at 300 nL min⁻¹ flow rate followed by a 5-minutes wash till 99% of solvent B. The MS settings were identical as previously described ⁷³. The immunoprecipitation-mass spectrometry data of temperature-treated Arabidopsis seedlings produced in this study have been deposited to the ProteomeXchange Consortium ⁷⁴ via the PRIDE partner repository with the dataset identifier PXD077449 (reviewers can access the dataset by logging in to the PRIDE website using the following account details: Username: reviewer_pxd077449@ebi.ac.uk /Password: s1o7a9rxlUxX). The spectra were searched using MaxQuant (version 1.5.7.0) on galaxy against a proteome database Araport11 ⁷⁵ for *A. thaliana*. The “Phospho (STY)Sites.txt”, “proteinGroups.txt”, and “peptides.txt” output files were analysed by Perseus (version 1.6.7.0).

Hypocotyl elongation assay

Seeds were surface sterilized and vertically grown on square plates with medium containing ½ Murashige-Skoog basal salt (½ MS) (Duchefa), 1% agar, and 1% sucrose. Seeds were stratified for 3 days at 4 °C firstly and then transferred to a growth chamber at 21 °C, with continuous light (70-100 µmol m⁻² s⁻¹) at 50% relative humidity for germination. After germination, seeds on vertical plates were transferred to short-day conditions of 8 h light/16 h dark either at 21 °C or 28 °C (100 µmol m⁻² s⁻¹ photosynthetically active radiation supplied by cool-white, fluorescent tungsten tubes, Osram). Plates were scanned at 7 days after germination. Measurement was done by using Fiji/ImageJ (<https://imagej.net/Fiji>).

Moderately high temperature stress tolerance assay

Arabidopsis moderately high temperature stress tolerance assays for *pAtCOI1::AtCOI1-GFP coi1-1*, *pAtCOI1::AtCOI1^{T16A}-GFP coi1-1*, *pAtCOI1::AtCOI1-GFP coi1-1 grxs17-1* and *pAtCOI1::AtCOI1^{T16A}-GFP coi1-1 grxs17-1* were performed using 7-day-old seedlings, seedlings were grown in plastic pots containing Jiffy Peat at 21 °C under the long-day condition (16h light/8h dark) for 7 days, and were subsequently transferred to an incubator set at 21 °C (control) or 37 °C for 4 days and allowed to recover at 21 °C for 10 days. In each biological replicate, at least 30 seedlings were used for each genotype. For thermotolerance assays in ½ MS solid medium, 7-day-old seedlings were grown on ½ MS with 0.8% agar, and 1% sucrose with long-day conditions of 16 h light/8 h dark at 21 °C (75 µmol m⁻² s⁻¹ photosynthetically active radiation supplied by light emitting diode (LED) lights). Plates were transferred to 21 °C (control) and 37 °C for 4 days under long-day conditions of 16 h light/8 h dark, and allowed to recover at 21 °C for 10 days. Moderately high temperature stress assays for *grxs17-1*, *grxs17-4* (*grxs17 Cas9-3*) and *grxs17 + GRXS17* genotypes, 7-day-old seedlings grown on ½ MS with 1% agar, and 1% sucrose with long-day conditions of 16 h light/8 h dark at 21 °C (75 µmol m⁻² s⁻¹ photosynthetically active radiation supplied by light-emitting diode (LED) lights). Plates were transferred to 21 °C (control) and 35 °C for 6 days under long-day conditions of 16 h light/8 h dark, and allowed to recover at 21 °C for 8 days. In wheat, to demonstrate the ultimate physiological output, (moderately) high temperature stress

tolerance assays were performed using 2-day-old seedlings that were grown in plastic pots containing soil at 21 °C under long-day conditions (16h light/8h dark), and that were transferred to an incubator set at 21°C (control) or 40 °C for 8 days or 37 °C for 10 days. Survival was confirmed through photographic evidence or visual scoring, identifying plants with damaged (wilted/brown) vs. surviving (green/regenerating) tissues. Survival rate (%) = (Number of surviving plants / total number of plants) × 100.

Moderately high temperature stress assays for ROS staining and H₂O₂ level quantification

Moderately high temperature stress assays were performed using 5-day-old seedlings, seedlings were grown at 21 °C in long-day conditions (16h light/8h dark) (75 µmol m⁻² s⁻¹ photosynthetically active radiation supplied by light emitting diode (LED) lights) on ½ MS with 0.8% agar solid medium, then 5-day-old seedlings were transferred to an incubator set at 21°C (control) or 37 °C for 2 days and allowed to recover at 21 °C in long-day conditions for 5 days. Seedlings were collected for ROS visualization. In parallel, the shoots were weighted, flash frozen, and ground into a powder with Retsh. Powdered samples are stored at -80 °C until analysis. Approximately 50-70 mg tissue per biological replicate was used for H₂O₂ extraction.

3,3'-diaminobenzidine (DAB) staining

For H₂O₂ visualisation, DAB (Sigma) staining solution was prepared by dissolving 50 mg of DAB in 50 ml of dH₂O for a final 1mg/ml concentration. The tubes were covered tube with aluminium foil since DAB is light-sensitive. 50 µl of TWEEN® 20 (0.1% v/v) was added to the solution and the pH was adjusted to < 3 with 37% HCl to facilitate DAB dissolution. DAB will still not fully dissolve, but usually very high levels of homogeneity in the solution are achieved, and the non-dissolved part is filtered out. pH was adjusted to 5.8 using 0.5 M Na₂HPO₄. Arabidopsis seedlings were vacuum-infiltrated in DAB solution for 20 minutes. After incubation in DAB solution at room temperature for 48 h in the dark, seedlings were briefly washed in dH₂O, and then bleached by boiling in 100% ethanol for 10 min in order to remove the chlorophyll and reveal the brown spots, which are indicative of the reaction of DAB with H₂O₂. After removing chlorophyll, seedlings were briefly washed in dH₂O. The stained seedlings can be immersed in dH₂O at 4°C for a few days until imaging analysis. The samples were observed and imaged under a microscope.

Nitroblue tetrazolium chloride (NBT) staining

For O₂⁻ visualisation, NBT (Duchefa) staining solution was prepared by dissolving 25 mg NBT in 50 ml of 1 × PBS (10mM PBS), pH was adjusted to 7.8. Arabidopsis seedlings were vacuum-infiltrated with NBT solution for 20 minutes. After incubation in NBT solution at room temperature for 24 h in the dark, seedlings were briefly washed in dH₂O, and then bleached by boiling in Lacto-glycerol-ethanol solution (1:1:4, lactic acid:glycerol:ethanol) for 15 min in order to remove the chlorophyll. The stained seedlings can be immersed in 80% lactic acid at 4 °C for weeks before imaging analysis. The samples were observed and imaged under a microscope.

H₂O₂ extraction and quantification.

Quantification of hydrogen peroxide was performed according to previous studies^{76–79} with some modifications. Briefly, 500 µl 1× phosphate buffer (pH 7.4) was added to finely ground shoot samples into a 2 ml Eppendorf tube. After the addition of phosphate buffer, the suspended tissues were vortexed for 10s and shaken continuously at 4°C for 30 min. Samples were centrifuged at 4°C for 10 min at 12000 × g. Supernatant was transferred to new tubes and stored on ice until the analysis was conducted on that day. The H₂O₂ level was measured using the Amplex Red kit. 154 µg of Amplex Red solid (one vial) is dissolved in 60 µl DMSO. 50 µl of dissolved Amplex Red is pipetted into 4.85 ml 1 × phosphate buffer (pH 7.4), and 100 µl horseradish peroxidase (HRP, 10 U/ml). So, the final volume of the Amplex Red reagent is 5 ml, and the final concentration is 24.94×10^{-6} M (~25 µM). To minimize the effect of potentially interfering substances (such as phenolics or pigments) in the plant extracts, the standard addition volume of the plant extract was optimized to a low volume (5 µl / well) in a 96-well plate format. Individual components were added to each well of the microfluor plate in the following order, for a total of 100 µl/well: 1× phosphate buffer (pH 7.4), 45 µl; H₂O₂ standard or plant sample (5 µl); Amplex Red reagent (50 µl). Following the addition of all components, the plate is covered using aluminium foil, shaken for 1 min at 450 rpm, and incubated at 30 °C for 120 min. For each plate, a blank (containing 50 µl phosphate buffer (pH 7.4) and 50 µl Amplex Red reagent) is run as a negative control. Stock H₂O₂ standards (2, 20, 50, 100 µM) are prepared in 1× phosphate buffer (pH 7.4) using freshly prepared 0.2 mM H₂O₂ working solution from a 3.0% (0.88 M) H₂O₂ stock solution. Standard calibration curves are prepared using 5 µl of the stock H₂O₂ standards (2, 20, 50, and 100 µM) to yield in-plate H₂O₂ concentrations of 0.1, 1, 2.5, and 5 uM, and are accepted if R²> 0.99. Microplate reader CLARIOstar equipped for excitation in the range of 530 – 560 nm and fluorescence emission detection at ~590 nm. To correct for background fluorescence, the fluorescence values of the no-H₂O₂ control were subtracted from each experimental sample. The H₂O₂ concentration of the plant extract was determined by interpolating the background-corrected values against the standard curve. The H₂O₂ content per gram of fresh weight was calculated by multiplying the concentration (M, equivalent to nmol/mL) by the total volume of the extract (e.g., ml) to find the total nanomoles in the sample and by then dividing the total nanomoles by the initial fresh weight (FW) of the plant tissue used for the extraction. Formula: nmol H₂O₂/g FW = [x nmol/ml × extract volume (ml)] / sample weight (g). It should be noted that slight discrepancies in absolute H₂O₂ levels or kinetics may arise from biological and technical factors. The complemented line used in this study was generated in the *coi1-1* mutant background rather than in a true wild-type Col-0 genetic background. Thus, although the *pAtCOI1::AtCOI1-GFP* transgene restores the major jasmonate- and temperature-responsive phenotypes of *coi1-1*, differences in the genetic background may contribute to variation in basal or stress-induced redox responses relative to native Col-0 plants.

Phylogenetics

The orthologues of Arabidopsis genes in other species (wheat, rice, and maize) were identified using gene trees available at EnsemblPlants. The amino acid sequences of the orthologs were aligned by the “Align by Muscle” function, and then the phylogenetic trees were reconstructed using an UPGMA statistical method implemented in MEGA7.0 and then manually edited.

Gene Ontology enrichment analyses and plot visualization

Gene Ontology (GO) enrichment analyses were performed using the PANTHER Classification System. GFP-AtCOI1 significantly enriched proteins (**Supplementary Tables S4 and S5**) identified at either 21 °C or 34 °C were analysed against all identified proteins (**Supplementary Table S3**) as the background set. Statistical significance was determined using Fisher’s exact test with a *p*-value cut-off of < 0.05. The GFP-AtCOI1 significantly enriched proteins were classified into four groups: (1) proteins enriched at either 21 °C or 34 °C (combined enriched proteins), (2) proteins commonly enriched at both 21 °C and 34 °C, (3) proteins specifically enriched at 21 °C, and (4) proteins specifically enriched at 34 °C. GO enrichment results from these four groups were merged into a single table (**Supplementary Table S6**) for downstream visualization. Bubble plots illustrating GO enrichment were generated in R using the packages dplyr, tidyr, stringr, and ggplot2, and the final figure layout was assembled in Inkscape.

***In silico* structural modelling of ternary complexes**

To investigate the structural architecture and interaction interfaces of the wild-type and mutant protein assemblies, three-dimensional structural models of the heteroternary complexes were generated. Full-length amino acid sequences for AtGRXS17 (Chain A), AtASK1 (Chain B), and AtCOI1 (Chain C) were obtained from The Arabidopsis Information Resource (TAIR). Predictions were conducted for the wild-type complex (AtGRXS17 + AtASK1 + AtCOI1) and the mutant variant (AtGRXS17 + AtASK1 + AtCOI1^{T16A}). Modelling was performed using AlphaFold 3 via the AlphaFold Server (<https://alphafoldserver.com>) under default multimer prediction settings. Predictions relied on standard multiple sequence alignments (MSAs) generated automatically against default databases, without custom templates. For each complex assembly, five independent structural models were sampled and ranked based on their overall confidence metrics.

Interface mapping and visual post-Processing

To comprehensively map the predicted intermolecular boundaries and evaluate the statistical confidence of the physical interactions, the highest-ranked AlphaFold 3 structural models were analysed using the AlphaBridge Webserver (alpha-bridge.eu). AlphaBridge was utilized to extract inter-chain interface contacts using default distance parameters, processing the structural coordinates and error matrices into unified, two-dimensional circular chord diagrams. Model prediction confidence was evaluated using the predicted local-distance difference test (pLDDT) per-residue score, categorized into four stringency brackets: Very High Confidence: (pLDDT > 90); Confident: 90 < pLDDT > 70; Low Confidence: 70 < pLDDT > 50; Very Low Confidence: pLDDT < 50. The central ribbons inside the chord diagrams delineate the confidently predicted inter-chain structural contacts connecting the respective subunits.

Data reproducibility

In order to assess the robustness of our results, some of the experiments were performed / repeated in different labs, with slightly different temperatures or settings, as indicated.

Statistical analyses

Phenotypic quantification, relative gene expression, and relative protein expression data were analysed using either One-way or Two-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) in GraphPad Prism 8 or 10. In some cases, data were analysed using Students' t-test with two-sample equal variance and one-tailed distribution. For box plots, plots with the individual show the median (central lines) with Tukey-based whiskers and outliers. The final figure layouts were assembled in Inkscape.

Declarations

Data availability

All data are available from the corresponding author upon request. Source data are provided with this paper. Mass spectrometry data of Arabidopsis are available via ProteomeXchange with identifier PXD077449

Reviewers can access the dataset by logging in to the PRIDE website using the following account details: Username: reviewer_pxd077449@ebi.ac.uk / Password: s1o7a9rxIUxX.

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Author contributions

T.Z., I.D.S., J.P.R. M. X., and A.G. designed and/or interpreted experiments. T.Z. performed most of the experiments. C.W., A.N.D., L.M. and L.P. generated tools. C.R. and J.P.R. performed some heat stress and

western-blot and analysed results. C.F.D.L generated wheat phosphoproteome datasets. I.V.L. performed one of the AtCOI1 and AtGRXS17 co-immunoprecipitation assays. B.V.D.C. prepared mass spectrometry samples and assisted with immunoprecipitation and selection of Arabidopsis transgenic lines. T.Z., A.G. and I.D.S. wrote the manuscript and all other authors edited or commented.

Competing interests

The authors declare no competing interests.

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Figures

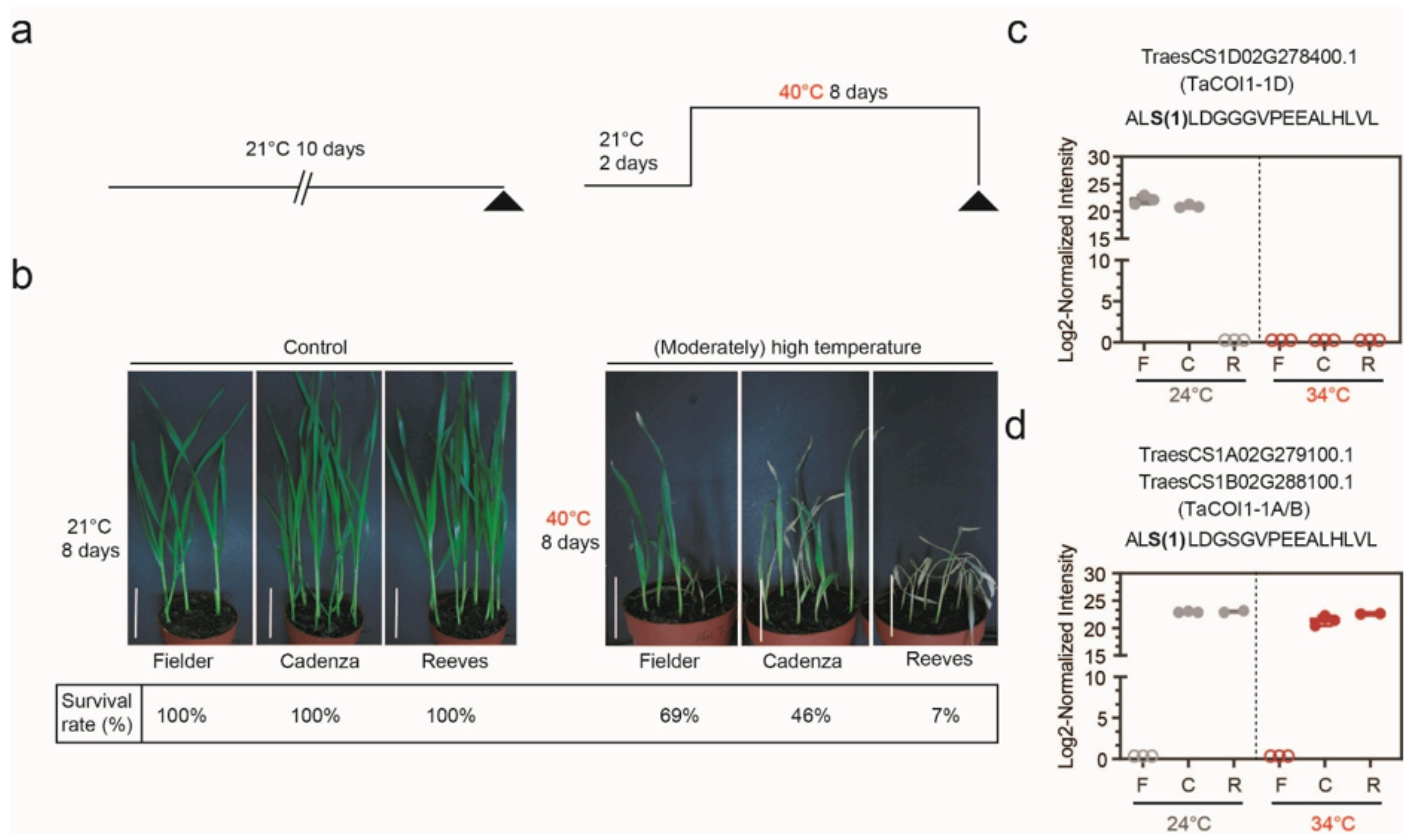


Figure 1

Identification of wheat COI1 phosphopeptides in different wheat varieties upon temperature treatment.

(a) Scheme of (moderately) high temperature assay conditions: control (left) and prolonged (moderately) high temperature treatment (right). Black triangle indicates the endpoint of treatment. (b) Representative images of (moderately) high temperature response of 10-day-old wheat varieties ‘Cadenza’, ‘Fielder’, and ‘Reeves’ wheat seedlings that were transferred to a growth chamber at 21°C or 40°C for 8 days to demonstrate the ultimate physiological output. Scale bar = 5 cm. The survival percentage was determined for control and (moderately) high temperature conditions (based on the number of living plants after treatment divided by the total number of plants initially placed under control or (moderately) high temperature) and shown below the images. At least 12 plants per genotype were examined. (c-d) Level of indicated phosphopeptide belonging to COI1 ortholog in ‘Fielder’ (F), ‘Cadenza’ (C) and ‘Reeves’ (R) wheat seedling leaves 7 days after germination when exposed to moderately high temperature (34°C) or control temperature (24°C) for 1 hour following growth at 24°C to capture the underlying molecular mechanism. 2-3 individual biological replicates (dots) displayed in the plot. Empty circle indicates the intensity is too low to detect. Data taken from our previously published data sets: ref. ³⁴ and ref. ³⁵.

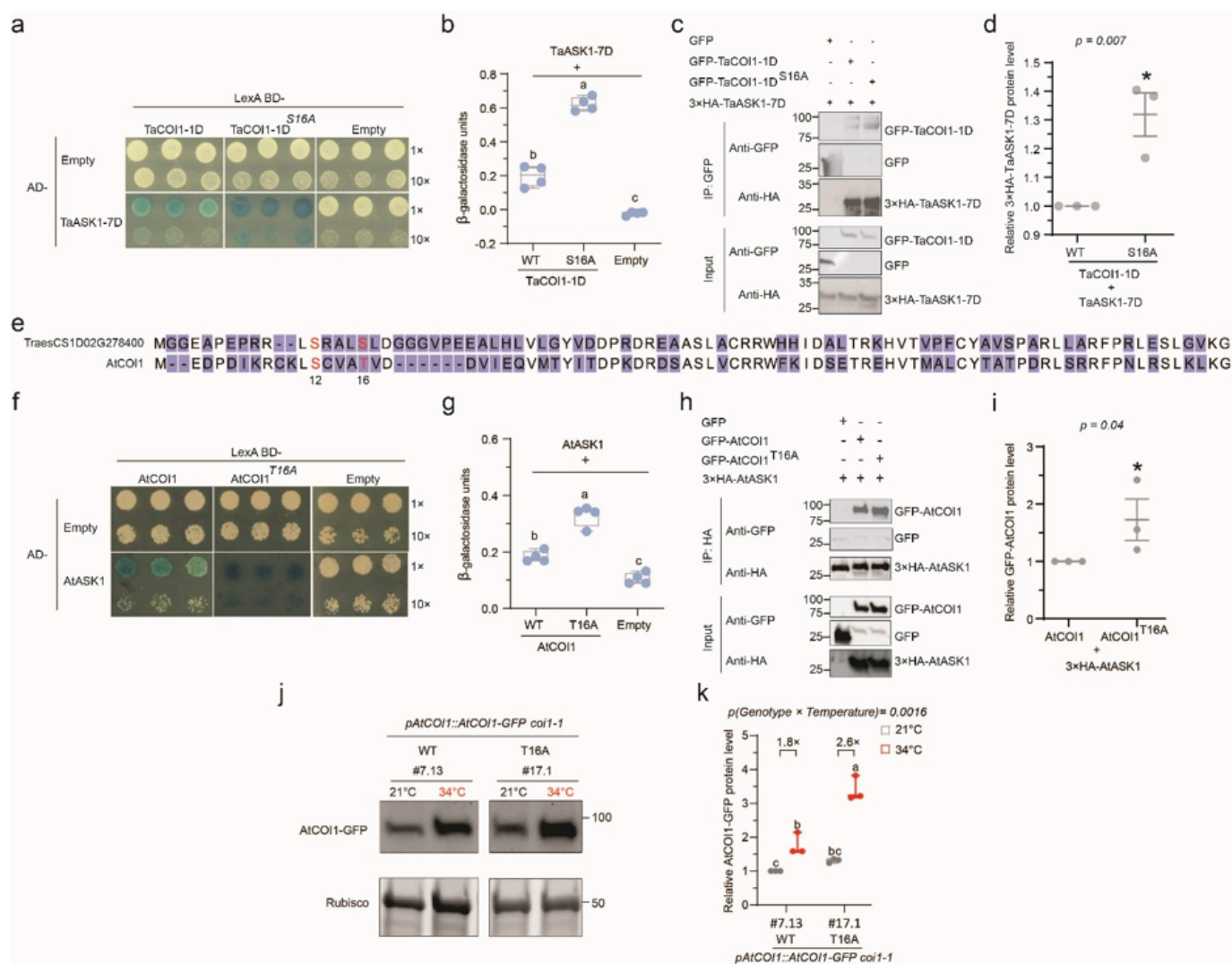
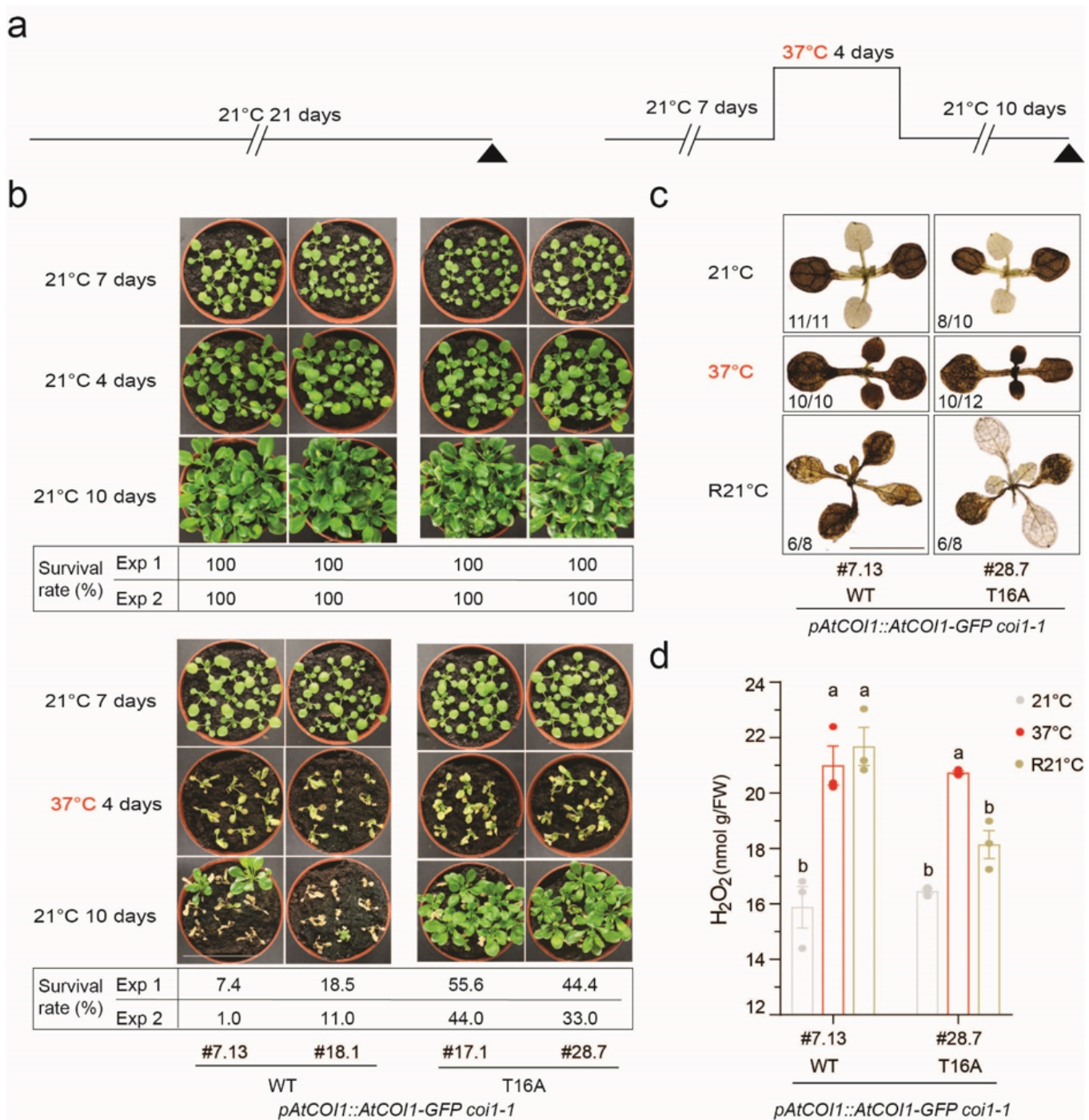


Figure 2

The Ser16 of TaCOI1-1D and Thr16 of AtCOI1 plays an essential role in enhancing COI1-ASK1 interaction and COI1 protein stability. (a-b) Yeast Two-Hybrid assay (a) and quantification of β -Galactosidase activity assay (b) for the interaction between TaCOI1-1D^{S16A} and TaASK1-7D. 1 $\times$ and 10 $\times$ represent the yeast liquid culture containing the corresponding proteins diluted to the indicated levels. Representative yeast colonies are shown, all independent colonies show similar interaction. LexA BD, LexA DNA-binding domain and AD, activation domain. Blue colony colour indicates positive interaction; white colony colour indicates no interaction. Box plots with individual data points show the distribution of all measurements, with the median (central lines), Tukey-based whiskers and outliers. The box edges show the first and third quartiles. The whiskers represent the range of data within 1.5 $\times$ the interquartile range from the first and third quartiles. Different letters denote significant differences ($p < 0.05$) based on one-way ANOVA with Tukey's HSD (b). (c-d) Representative Western blot (c) and quantification (d) of

TaASK1-7D from *Nicotiana benthamiana* leaves transiently co-expressing GFP, GFP-TaCOI1-1D or GFP-TaCOI1-1D^{S16A} with 3×HA-TaASK1-7D. The immunocomplex and input proteins were analysed by immunoblotting using anti-HA and anti-GFP antibodies. The experiments were independently repeated 3 times (individual dots in the plot) with similar results, and relative 3×HA-TaASK1-7D protein levels were quantified and normalized to GFP-enriched GFP-TaCOI1-1D in three independent experiments (d). An * denotes a significant difference ($p < 0.05$) using a student's t-test with two-sample equal variance and one-tailed distribution. The exact p -value is shown at the top. **(e)** Protein sequence alignment of the N-terminal part of wheat COI1-1D (TraesCS1D02G278400) and Arabidopsis COI1. Potentially conserved phosphosites Serine 12 and Threonine 16 are marked in red. **(f-g)** Yeast Two-Hybrid assay (f) and quantification of β -Galactosidase activity assay (g) for the interaction of AtCOI1 or AtCOI1^{T16A} with AtASK1. 1× and 10× represent the yeast liquid culture containing the corresponding proteins diluted to the indicated concentrations. Representative yeast colonies are shown, all independent colonies show similar interaction. Blue colony colour indicates positive interaction; white colony colour indicates no interaction. LexA BD, LexA DNA-binding domain and AD, activation domain. Box plots with individual data points represent the distribution of all individual measurements, showing the median (central lines) with Tukey-based whiskers and outliers. The box edges show the first and third quartiles. The whiskers represent the range of data within 1.5× the interquartile range from the first and third quartiles. Different letters denote significant differences ($p < 0.05$) based on one-way ANOVA with Tukey's HSD (h). **(h-i)** Representative Western blot (h) and quantification (i) of AtCOI1 or AtCOI1^{T16A} and AtASK1 from *Nicotiana benthamiana* leaves transiently co-expressing GFP, GFP-AtCOI1 or GFP-AtCOI1^{T16A} with 3×HA-AtASK1. The immunocomplex and input proteins were analysed by immunoblotting using anti-HA and anti-GFP antibodies. Relative GFP-AtCOI1 protein levels were quantified and normalized to HA-enriched 3×HA-AtASK1 in three independent experiments (individual dots in the plot) with similar results (i). An * denotes a significant difference ($p < 0.05$) using a student's t-test with two-sample equal variance and one-tailed distribution. The exact p -value is shown at the top. **(j-k)** Representative Western blot (j) and quantification (k) showing AtCOI1-GFP levels in *pAtCOI1::AtCOI1-GFP coi1-1* (#7.13) and *pAtCOI1::AtCOI1^{T16A}-GFP coi1-1* (#17.1) seedlings treated for 5 hours at 21°C and 34°C and probed with an anti-GFP antibody. Stain-free gel highlighting Rubisco as a loading control. The experiment was repeated three times (individual dots in the plot) with similar results. Relative AtCOI1-GFP protein levels were plotted (k). Box plots with individual data points show the distribution of all measurements, with the median (central lines), Tukey-based whiskers and outliers. The box edges show the first and third quartiles. The whiskers represent the range of data within 1.5× the interquartile range from the first and third quartiles. Different letters denote significant differences ($p < 0.05$) based on two-way ANOVA with Tukey's HSD, with fold-change between 21°C and 34°C per genotype. The exact p -value for the interaction (Genotype × Temperature) (k) is shown at the top.



experiments (Exp1 and Exp 2) are shown. The survival percentage was determined for control and moderately high temperature conditions (based on the number of living plants after recovery divided by the total number of plants initially placed under control or moderately high temperature) and shown below the images. In each experiment, at least 30 plants per genotype were examined. Scale bar, 5 cm. **(c)** Representative images of 3,3'-diaminobenzidine (DAB) staining of 7-day-old *pAtCOI1::AtCOI1-GFP coi1-1* (#7.13) and *pAtCOI1::AtCOI1^{T16A}-GFP coi1-1* (#28.7) seedlings grown at 21°C, 37°C and recovery 5 days at 21°C after 37°C. The number of seedlings that show a similar pattern to the representative image is indicated in the image. Scale bar, 5 mm. **(d)** H₂O₂ level was measured by Amplex™ Red Hydrogen Peroxide kit using the samples from the same experiment as DAB staining used in (c). The bar plot shows the value of the 3 individual biological replicates (dots) with the error bars ("± SEM"). Each biological replicate was pooled with around 50 mg of seedlings. Different letters denote significant differences ($p < 0.05$) based on two-way ANOVA with Tukey's HSD.

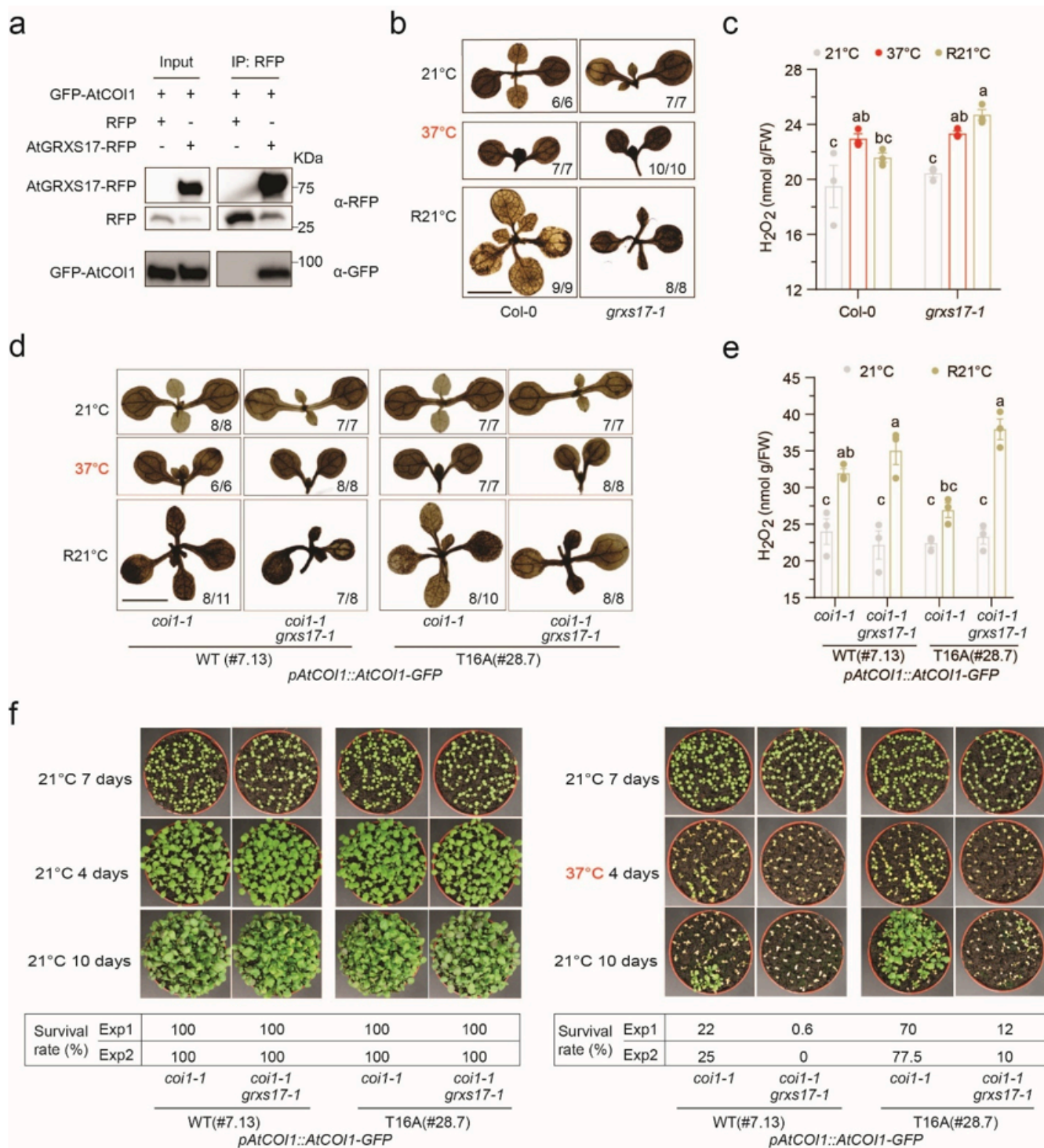


Figure 4

Arabidopsis COI1 interacts with GRXS17 to modulate H₂O₂ removal and thermotolerance. (a)

Representative Western blot of Arabidopsis COI1 and GRXS17 from *Nicotiana benthamiana* leaves transiently co-expressing GFP or GFP-AtCOI1 with RFP or AtGRXS17-RFP. The immunocomplex and input proteins were analysed by immunoblotting using anti-GFP and anti-RFP antibodies. The experiments were independently repeated 3 times with similar results. (b) Representative images of 3,3'-

diaminobenzidine (DAB) staining of 7-day-old wild-type Col-0 and *grxs17-1* seedlings grown at 21°C, 37°C, and recovery for 5 days at 21°C after 37°C. The number of seedlings that show a similar pattern to the representative image is indicated in the image. Scale bar, 5 mm. (c) H₂O₂ level using seedlings from the experiment in (b). Bar plot shows the value of the 3 individual biological replicates (dots) with the error bars (± SEM). Different letters denote significant differences ($p < 0.05$) based on two-way ANOVA with Tukey's HSD. (d) Representative images of 3,3'-diaminobenzidine (DAB) staining of 7-day-old *pAtCOI1::AtCOI1-GFP coi1-1* or *pAtCOI1::AtCOI1-GFP coi1-1 grxs17-1* and *pAtCOI1::AtCOI1^{T16A}-GFP coi1-1* or *pAtCOI1::AtCOI1^{T16A}-GFP coi1-1 grxs17-1* seedlings grown at 21°C, 37°C and recovery 5 days at 21°C after 37°C. The number of seedlings that show a similar pattern as the representative image indicated on the image. Scale bar, 5 mm. (e) H₂O₂ level in seedlings from the experiment in (d). Bar plot shows the value of the 3 individual biological replicates (dots) with the error bars ("± SEM"). Different letters denote significant differences ($p < 0.05$) based on two-way ANOVA with Tukey's HSD. (f) Representative images of *pAtCOI1::AtCOI1-GFP coi1-1* or *pAtCOI1::AtCOI1-GFP coi1-1 grxs17-1* and *pAtCOI1::AtCOI1^{T16A}-GFP coi1-1* or *pAtCOI1::AtCOI1^{T16A}-GFP coi1-1 grxs17-1* seedlings under control and prolonged moderately high temperature treatment. Results from one representative experiment of two independent experiments (Exp 1 and Exp 2) are shown. The survival percentage was determined for control and moderately high temperature conditions (based on the number of living plants after recovery divided by the total number of plants initially placed under control or moderately high temperature) and shown below the images. In each experiment, at least 39 plants per genotype were examined.

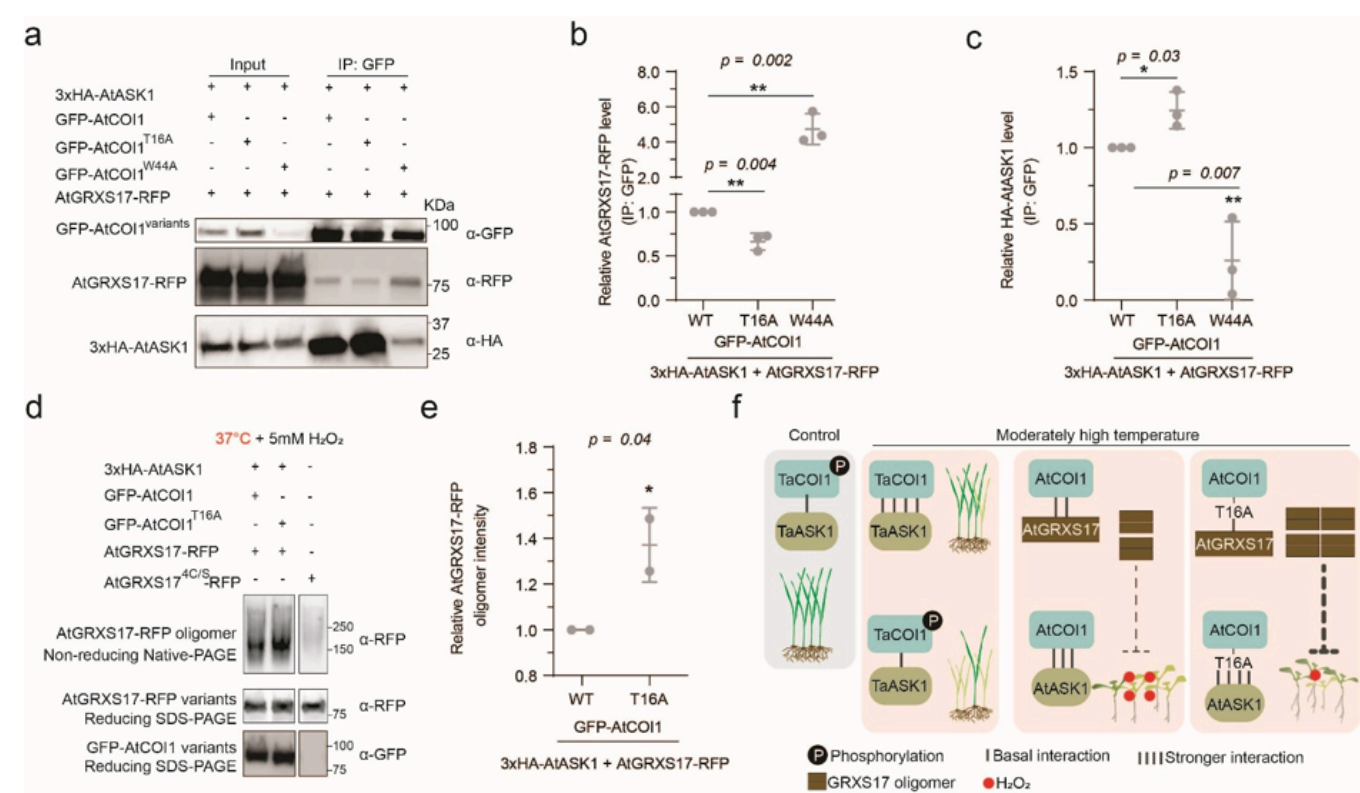


Figure 5

Arabidopsis COI1^{T16A} variant reduces affinity with GRXS17 to release GRXS17 for modulating H₂O₂ level and thermotolerance. (a-c) Representative Western blot (a) and quantification (b-c) of AtASK1, AtCOI1, AtCOI1^{T16A} or AtCOI1^{W44A} and AtGRXS17 from *Nicotiana benthamiana* leaves transiently co-expressing GFP, GFP-AtCOI1, GFP- AtCOI1^{T16A} or GFP- AtCOI1^{W44A} with RFP or AtGRXS17-RFP and 3×HA-AtASK1. The GFP-trap incubated immunocomplex and input proteins were analysed by immunoblotting using anti-GFP, anti-RFP, and anti-HA antibodies. Relative AtGRXS17-RFP (b) and HA-AtASK1 (c) protein levels were quantified using three independent experiments (a). * denotes significant differences ($p < 0.05$) based on Students' t-test with two-sample equal variance and one-tailed distribution. (d-e) Western blot of AtGRXS17-RFP analysed by non-reducing Native PAGE. Proteins from *Nicotiana benthamiana* leaves transiently co-expressing AtGRXS17-RFP with GFP-AtCOI1 or GFP-AtCOI1^{T16A} and 3×HA-AtASK1, AtGRXS17^{4C/S}-RFP used as control. Following confirmation of robust protein expression by confocal laser scanning microscopy, the leaves were infiltrated with 5 mM H₂O₂. The plants were immediately treated with 37 °C for 5 hours. Total proteins were analysed by immunoblotting using anti-RFP and anti-GFP antibodies. * denotes significant differences ($p < 0.05$) based on Students' t-test with two-sample equal variance and one-tailed distribution. (f) Proposed working model for the role of COI1 phosphorylation status in the regulation of the ASK1-COI1-GRXS17 module, mediating the redox responses required for plant thermotolerance. Dashed line represents possible direct or indirect effect.

Supplementary Files

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