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Integration of stress-related and reactive oxygen species-mediated signals by Topoisomerase VI in *Arabidopsis thaliana*

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Environmental stress often leads to an increased production of reactive oxygen species that are involved in plastid-to-nucleus retrograde signaling. Soon after the release of singlet oxygen ($^1\text{O}_2$) in chloroplasts of the *flu* mutant of *Arabidopsis*, reprogramming of nuclear gene expression reveals a rapid transfer of signals from the plastid to the nucleus. We have identified extraplastidic signaling constituents involved in $^1\text{O}_2$ -initiated plastid-to-nucleus signaling and nuclear gene activation after mutagenizing a *flu* line expressing the luciferase reporter gene under the control of the promoter of a $^1\text{O}_2$ -responsive AAA-ATPase gene (*At3g28580*) and isolating second-site mutations that lead to a constitutive up-regulation of the reporter gene or abrogate its $^1\text{O}_2$ -dependent up-regulation. One of these mutants, *caa39*, turned out to be a weak mutant allele of the Topoisomerase VI (Topo VI) A-subunit gene with a single amino acid substitution. Transcript profile analysis of *flu* and *flu caa39* mutants revealed that Topo VI is necessary for the full activation of AAA-ATPase and a set of $^1\text{O}_2$ -responsive transcripts in response to $^1\text{O}_2$. Topo VI binds to the promoter of the AAA-ATPase and other $^1\text{O}_2$ -responsive genes, and hence could directly regulate their expression. Under photoinhibitory stress conditions, which enhance the production of $^1\text{O}_2$ and H_2O_2 , Topo VI regulates $^1\text{O}_2$ -responsive and H_2O_2 -responsive genes in a distinct manner. These results suggest that Topo VI acts as an integrator of multiple signals generated by reactive oxygen species formed in plants under adverse environmental conditions.

oxidative stress | light stress | cell death

Plants are often exposed to environmental changes that adversely affect their growth and development and may ultimately result in the death of the plant. Most of these stress conditions disrupt the metabolic balance of cells and increase the production of reactive oxygen species (ROS) (1). ROS may be toxic and cause oxidative damage, or they may act as signaling molecules and activate the plant's defenses against environmental stress (2, 3). The specificity of these responses is largely determined by the chemical identity of the ROS (4, 5). Research in the past has primarily been concerned with studying the biological activities of superoxide anion ($\text{O}_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (6, 7), whereas the analysis of singlet oxygen ($^1\text{O}_2$) and hydroxyl radical ($^{\bullet}\text{OH}$) has been impeded by the lack of suitable experimental systems and detection techniques. Only recently, by using the conditional *flu* mutant of *Arabidopsis thaliana*, has $^1\text{O}_2$ been shown to be involved in plastid-to-nucleus retrograde signaling. Immediately after the onset of $^1\text{O}_2$ generation in plastids of *Arabidopsis*, changes in nuclear gene expression reveal a rapid transfer of $^1\text{O}_2$ -derived signals from the plastid to the nucleus (8–10). Several $^1\text{O}_2$ -responsive genes are different from those activated by $\text{O}_2^{\bullet-}$ or H_2O_2 , suggesting that $\text{O}_2^{\bullet-}/\text{H}_2\text{O}_2$ - and $^1\text{O}_2$ -dependent signaling occurs via distinct pathways (8, 10, 11).

Other consequences of increased $^1\text{O}_2$ generation inside plastids include a drastic reduction in the growth rate of mature plants and the bleaching and death of seedlings (10). All these $^1\text{O}_2$ -mediated responses are genetically regulated by the two plastid proteins, EXECUTER1 and EXEXUTER2, required for the translocation of $^1\text{O}_2$ -derived signals from the plastid to the nucleus (12, 13).

$^1\text{O}_2$ signaling does not seem to operate via an isolated signaling pathway but rather as part of a complex signaling network that integrates various extra- and intracellular cues (14). We previously used a genetic approach to penetrate this complexity. A transgenic *flu* line expressing an $^1\text{O}_2$ -responsive reporter gene was mutagenized, and we isolated second-site mutations that either led to a constitutive up-regulation of the reporter gene or abrogated its $^1\text{O}_2$ -dependent up-regulation (14). The reporter gene consisted of the luciferase ORF and the promoter of the $^1\text{O}_2$ -responsive AAA-ATPase nuclear gene of *Arabidopsis* (8, 10). Here we report on the identification and characterization of mutant *caa39*, whose response to $^1\text{O}_2$ is impaired. We found that *caa39* is a weak mutant allele of the Topoisomerase VI (Topo VI) A-subunit (*AtTOP6A*) gene with a single amino acid substitution. Under photoinhibitory stress conditions, *AtTOP6A* is indispensable for the selective activation of several $^1\text{O}_2$ -responsive nuclear genes and at the same time may act as a repressor of H_2O_2 -responsive genes. This dual activity assigns a key role to Topo VI as an integrator of multiple ROS signals that are released by plants in response to adverse environmental conditions.

Results

Isolation and Characterization of the *caa39* Mutant. The *Arabidopsis flu AAA:LUC⁺* line, which expresses the luciferase (LUC) reporter gene under the control of the $^1\text{O}_2$ -responsive AAA-ATPase promoter (14), was mutagenized and screened for second-site mutants that either constitutively up-regulate the $^1\text{O}_2$ -responsive reporter gene or have lost the ability to respond

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to $^1\text{O}_2$. Six *flu* *AAA:LUC*⁺ *caa* mutants were isolated that in 10-d-old seedlings constitutively activate both the reporter gene and the endogenous *AAA-ATPase* gene, and hence carry *trans*-acting mutations (14). In *caa* mutants with mutations in genes that genetically form part of the $^1\text{O}_2$ -signaling pathway and that also contain the *flu* mutation, generation of $^1\text{O}_2$ by a dark-to-light (D/L) shift should not further enhance the constitutive expression of $^1\text{O}_2$ -responsive genes. Based on this criterion, only one of the six *caa* mutants, *caa39*, could be directly linked to $^1\text{O}_2$ signaling (14–16). Until 5 d old, *caa39* mutant seedlings grown under continuous light were phenotypically similar to wild-type seedlings (Fig. 1A) and E).

CAA39 Encodes the A-Subunit of Arabidopsis Topo VI. The visible morphological alterations and constitutive expression of the *AAA:LUC*⁺ reporter gene cosegregated as a single recessive Mendelian trait when crossed with the parental *flu* *AAA:LUC*⁺ line (14). By map-based cloning, the *caa39* mutation could be assigned to a fragment of ~90 kb covered by the two BAC clones, F9G14 and F15A17 (Fig. 1C and Fig. S1). This region is predicted to contain 22 ORFs (<http://www.tigr.org>). By sequencing DNA in this region we identified a single G-to-A nucleotide substitution in locus *At5g02820* of *flu* *AAA:LUC*⁺ *caa39* relative to the parental and wild-type lines (Fig. 1D). *At5g02820* encodes the Topo VI A-subunit AtTOP6A/AtSPO11-3/RHL2/BIN5, which is homologous to the eukaryotic SPO11 meiotic recombination endonuclease. The *caa39* mutation results in the conversion of proline to leucine at position 337 within the topoisomerase-primase domain (Fig. 1D). Proline 337 is highly conserved in TOP6A/SPO11-3 homologs (Fig. S2). The identification of *caa39* was confirmed by complementing the mutant with the complete ORF of *AtTOP6A* under the control of the cauliflower mosaic virus 35S promoter and by allelism tests. Complemented *flu* *caa39* 35S:*AtTOP6A* plants were phenotypically indistinguishable from the parental *flu* plants, and *AAA-ATPase* transcript levels returned to the low basal level found in light-grown *flu* (Fig. 1E). For allelism tests, *caa39* was crossed with the allelic mutant *rhl2-1* (17). F1 seedlings from this cross retained high LUC activity and displayed a phenotype intermediate between the parental lines (Fig. S3). Significantly, in *rhl2-1* seedlings expression of the endogenous *AAA-ATPase* was also constitutively up-regulated to a similar high level as in *caa39*, despite a more severe morphological phenotype (Fig. 2).

Topo VI is an ATP-dependent type II topoisomerase (type IIB) enzyme found in archaea, plants, red algae, diatoms, and a few protists (18). Archaea Topo VI forms an A₂B₂ heterotetramer (19). In *Arabidopsis* the B subunit, which is closely related to the ATPase region of type IIA topoisomerases (20, 21), is encoded by a single gene, *At3g20780* *AtTOP6B/RHL3/BIN3/HYP6*. In addition to the homologs of the archaeal Topo VI A and B subunits, *Arabidopsis* Topo VI also contains two small subunits, ROOT HAIRLESS 1 (RHL1) (22)/HYPOCOTYL 7 (HYP7) (23) and BRASSINOSTEROID-INSENSITIVE 4 (BIN4) (24)/MIDGET (MID) (25). If the regulatory role of AtTOP6A is linked to its function as a subunit of the *Arabidopsis* Topo VI complex, deletion of either subunit should have the same effect as in the *caa39* mutant. Therefore, we analyzed mutants for each protein in the plant Topo VI complex and for two *AtTOP6A* homologs that are not associated with Topo VI but are required for meiotic recombination [*AtSPO11-1* (*spo11-1-3*) and *AtSPO11-2* (*spo11-2*)] (26). These mutants were grown for 10 d under the same continuous light conditions together with *flu* *caa39*, *flu*, and wild-type Col. *spo11-1-3* and *spo11-2* mutant plants were morphologically indistinguishable from wild-type, whereas mutants in subunits of the Topo VI complex were severely affected in their growth and showed chlorotic cotyledons (Fig. 2). Seedlings of *caa39* displayed a much less severe phenotype. Inactivation of different subunits of the Topo VI complex resulted in very similar expression signatures (constitutive up-regulation of the $^1\text{O}_2$ -responsive genes

AAA-ATPase and *BAP1*, whereas the H_2O_2 -responsive marker gene *FER1* was not or barely affected) despite the fact that *caa39* growth phenotype is much less severe than in the *AtTOP6A* knockout mutants or the other Topo VI mutants (Fig. 2). In contrast, impairment of the SPO11 meiotic recombination endonuclease function in *spo11-1-3* and *spo11-2* mutants resulted in very different expression profiles (Fig. 2). These results support the notion that AtTOP6A modulates $^1\text{O}_2$ -responsive gene expression while being part of the Topo VI complex.

AtTOP6A Regulates $^1\text{O}_2$ -Triggered Stress Responses. To confirm that AtTOP6A represents a genuine $^1\text{O}_2$ signaling component, we further analyzed the *flu* *caa39* mutant under $^1\text{O}_2$ -producing conditions. As cotyledons of 10-d-old *flu* seedlings grown under continuous light are no longer able to accumulate significant amounts of the photosensitizer protochlorophyllide (Pchl_{id}) in the dark and, hence, do not release $^1\text{O}_2$ during reillumination, this analysis was performed in 5-d-old seedlings. The expression of *LUC* and the endogenous *AAA-ATPase* was only moderately enhanced in 5-d-old *flu* *caa39* seedlings under steady-state conditions relative to *flu* (Fig. 1A and B). Following a D/L shift, $^1\text{O}_2$ -induced accumulation of *AAA-ATPase* and *BAP1* transcripts was strongly suppressed in *flu* *caa39* seedlings (Fig. 3A), even though they accumulated similar excess amounts of Pchl_{id} as the parental *flu* line (14); a full induction was restored in complemented *flu* *caa39* 35S:*AtTOP6A* plants (Fig. 3A). The impaired $^1\text{O}_2$ -induced accumulation of *AAA-ATPase* transcripts in 5-d-old *flu* *caa39* seedlings after a D/L shift could not be attributed to saturated expression of *AAA-ATPase* before $^1\text{O}_2$ production because: (i) 5-d-old *flu* *caa39* seedlings only moderately accumulated *AAA-ATPase* transcripts compared with 10-d-old seedlings (Fig. 1B), and (ii) other *flu* *caa* mutants that constitutively accumulated similar or higher amounts of *AAA-ATPase* transcripts than *flu* *caa39* were still able to further accumulate *AAA-ATPase* transcripts in response to $^1\text{O}_2$ in 5-d-old seedlings (14–16).

It is conceivable that Topo VI may also regulate the expression of other $^1\text{O}_2$ -responsive genes and affect phenotypic changes, such as cell death, that are triggered by the release of $^1\text{O}_2$ in the *flu* mutant (10). Therefore, we tried to identify genes that were affected by the *caa39* mutation before and after the release of $^1\text{O}_2$. Genome-scale gene expression profiling was performed using Affymetrix *Arabidopsis* AGRONOMICS1 microarrays (<http://www.agron-omics.eu>); 5-d-old *flu* and *flu* *caa39* seedlings were grown under continuous light, transferred to darkness for 8 h, and reilluminated for 30 min to generate $^1\text{O}_2$. Based on the expression data of biological triplicates and a selection criterion of 1.5-fold change, 1,093 genes were identified that exhibited reproducible activation or repression in *caa39* or after a D/L shift in *flu*. The *AAA-ATPase*, the very low basal expression level of which exceeded the background level on the microarrays only in *flu* after reillumination, was not retained in this analysis. K-means clustering was performed to identify genes that may form coregulated clusters. Eight different gene clusters could be clearly distinguished (Fig. 3B). Clusters 1 and 2 principally comprised genes that are constitutively up-regulated and down-regulated, respectively, in *caa39*, but not affected by a D/L shift in *flu*. Cluster 1 is highly enriched in genes related to DNA repair/response to DNA damage stimulus, such as *ATMND1*, *RAD51*, *BRCA1*, *XRI1*, *PARP2*, *ATRAD17*, and *TSO2* [cluster frequency 15.2%, background TAIR (The *Arabidopsis* Information Resource database, www.arabidopsis.org) frequency 0.6%, *P* value 2.31 $\times 10^{-6}$] (Dataset S1), indicating that although no cell death could be detected in 5-d-old *flu* *caa39* seedlings under continuous light (Fig. 3C), the Topo VI mutation *caa39* causes DNA damages, the accumulation of which will eventually lead to the appearance of cell death at later stages. Conversely, such a DNA damage response is not activated in the *flu* mutant following the release of $^1\text{O}_2$. Cluster 4 comprised genes that are up-regulated after a D/L shift in *flu* and less induced in the *flu* *caa39* mutant, but not affected by the *caa39* mutation before the D/L shift (i.e., genes that are induced by $^1\text{O}_2$

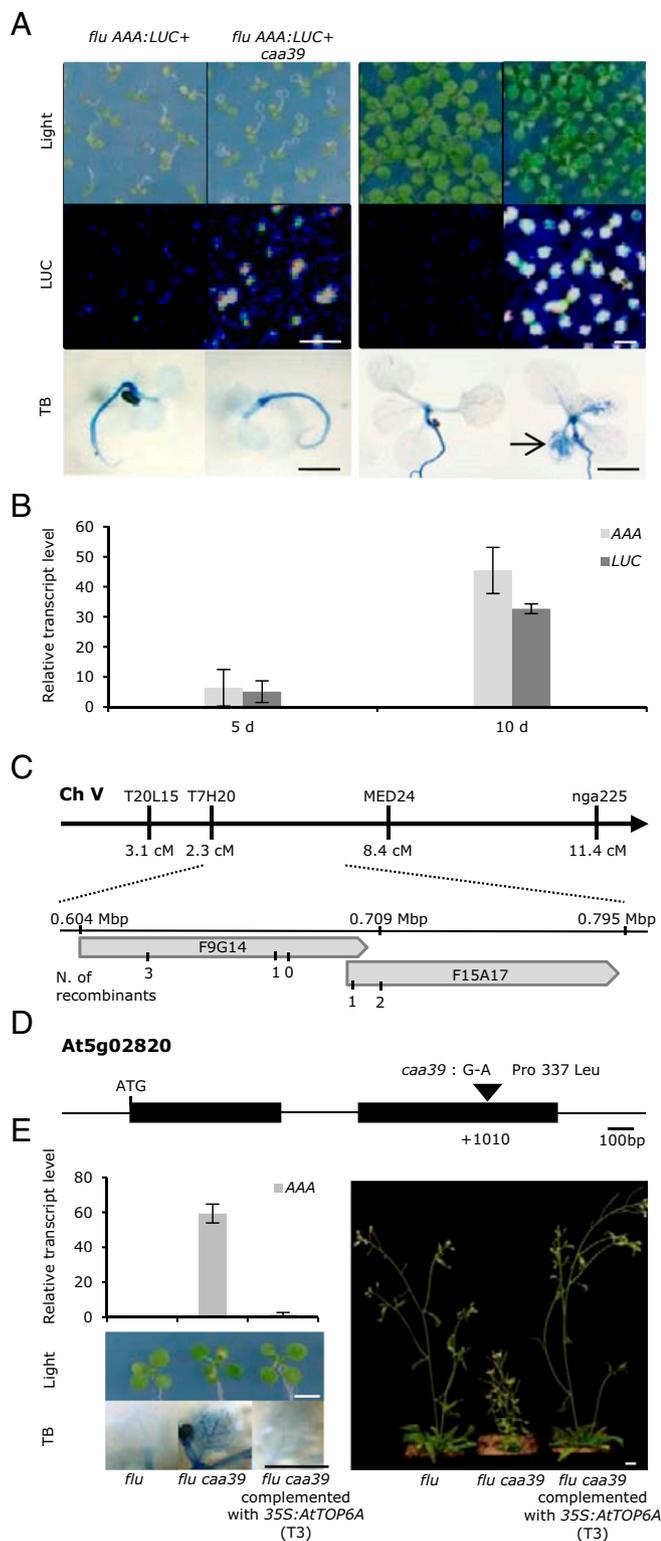


Fig. 1. Identification of the CAA39 gene. (A) Constitutive luciferase activity and phenotype of the *flu* AAA:*LUC*⁺ *caa39* and the *flu* AAA:*LUC*⁺ parental line. Seedlings were grown in continuous light on Murashige and Skoog agar plates for 5 d and 10 d. Bioluminescence (LUC), corresponding visible images (Light), and Trypan blue (TB) staining of cell death are presented. (Scale bars, 0.5 cm.) (B) Relative transcript levels of the AAA-ATPase gene (AAA) and the Luciferase reporter gene (*LUC*) in cotyledons of 5-d-old and 10-d-old seedlings of the *flu* AAA:*LUC*⁺ *caa39* mutant and the *flu* AAA:*LUC*⁺ parental line. Transcript levels were determined by quantitative RT-PCR. Results represent mean values of two biological replicates \pm SE. (C) Map-

ing in a Topo VI-dependent manner) (Fig. 3B and Dataset S1). Conversely, genes in cluster 5 (Fig. 3B and Dataset S1) are further activated in *flu caa39* following a D/L shift; interestingly, this cluster contains four two-component response regulator genes (cluster frequency 7.3%, background TAIR frequency 0.1%, P value 1.99×10^{-4}). Clusters 6 and 8 comprised genes that are up-regulated in *caa39* both before and after a D/L shift in *flu*. Finally, the main cluster (cluster 7) consists of 398 genes that are activated after a D/L shift and do not seem to be regulated by AtTOP6A (Fig. 3B and Dataset S1). Similarly, genes that are down-regulated after a D/L shift are not or barely affected by the *caa39* mutation (cluster 3). Collectively, these expression data identify sub-fractions of $^1\text{O}_2$ -responsive genes that are controlled by Topo VI, either in a positive or negative manner. When we examined the phenotypic responses of *flu* AAA:*LUC*⁺ *caa39* to $^1\text{O}_2$ generated by a D/L shift, we found that the cell-death response was significantly reduced compared with the parental line (Fig. 3C and D).

Contrasting Roles of AtTOP6A During Expression Changes of $^1\text{O}_2$ - and H_2O_2 -Responsive Genes Under High Light Stress. The physiological role of AtTOP6A was further assessed in wild-type and *caa39* plants exposed to a high light treatment that causes photo-inhibition and increases the production of $^1\text{O}_2$ and H_2O_2 (27). We analyzed the expression of the AAA-ATPase and other $^1\text{O}_2$ -induced genes that are selectively activated by $^1\text{O}_2$ from the three main clusters [i.e., *Atlg24145* from cluster 4, *BAP1* from cluster 6 (10) and *ERF5* from cluster 7 (28, 29)], as well as three H_2O_2 -responsive genes [i.e., *FER1*, *APX1*, and *At3g49160* (*PK*, pyruvate kinase-like)], and finally *ZAT12*, which was shown to be induced in several ROS-producing conditions (29). Seedlings were initially grown for 5 d under $80\text{-}\mu\text{mol photons m}^{-2}\text{s}^{-1}$ before transferring them to high light ($2,000\text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). In wild-type plants, transcripts of the $^1\text{O}_2$ -responsive genes rapidly and transiently accumulated first within 15–30 min after the start of high light treatment (Fig. 4), but the induction of H_2O_2 -responsive genes was weaker and delayed. Expression of the general oxidative stress-response gene *ZAT12* displayed an intermediate kinetic relative to that of the $^1\text{O}_2$ and H_2O_2 -responsive genes (Fig. 4). In *caa39*, induction of the $^1\text{O}_2$ -responsive genes was reduced (Fig. 4), except *ERF5* that was also unaffected by the *caa39* mutation in the *flu* mutant (Fig. 3B, cluster 7). Conversely, the *caa39* mutation augmented the stress-induced activation of the H_2O_2 -responsive genes *FER1* and *PK*, but hardly affected *APX1*. Very similar induction patterns and effects of the *caa39* mutation were observed in an experiment where the original *flu* AAA:*LUC*⁺ and *flu* AAA:*LUC*⁺ *caa39* lines were grown for 6 d under continuous low light (when they reach a developmental stage similar to seedlings grown for 5 d under continuous normal light) before transferring them to continuous moderate high light ($1,050\text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) (Fig. S4). These results suggest that AtTOP6A can act as a positive regulator of $^1\text{O}_2$ -responsive genes and simultaneously suppress the expression of some H_2O_2 -responsive genes under photoinhibitory stress conditions that generate both ROS.

Topo VI Directly Binds to the Proximal Promoter Region of $^1\text{O}_2$ -Responsive Genes. Topo VI could regulate gene expression either directly by binding to the promoter of a particular target gene or indirectly. Therefore, we used ChIP assays to examine

based cloning of CAA39. Initial mapping analysis revealed genetic linkage of CAA39 to markers located on top of chromosome V. Fine mapping localized the *caa39* mutation in a 100-kb region covered by BAC clones F9G14 and F15A17. (D) A single G-to-A nucleotide substitution was found in the second exon of the *At5g2820* locus in the *caa39* mutant, resulting in a Pro-337 to Leu amino acid exchange. Filled boxes indicate exons, lines indicate transcribed regions. (E) Complementation of *caa39*. AAA-ATPase expression, morphological phenotype and Trypan blue staining of cell death in 10-d-old seedlings, (Left) and morphological phenotype of 35-d-old mature plants (Right) of *flu*, *flu caa39*, and *flu caa39* complemented with the wild-type copy of the *At5g2820* gene. (Scale bars, 0.5 cm.)

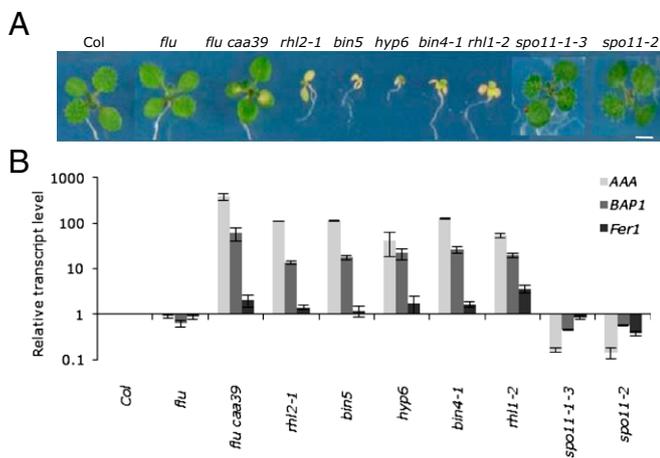


Fig. 2. Phenotype of mutants with impaired *Arabidopsis* Topo VI subunits. (A) Visible phenotypes of 10-d-old seedlings grown under continuous light. (B) Relative transcript levels of endogenous *AAA-ATPase* (*AAA*), *BAP1* and *FER1* marker genes were analyzed in cotyledons of 10-d-old seedlings by quantitative RT-PCR and compared with Col. *spo11-1-3* and *spo11-2* were included as controls. Results represent mean values of two biological replicates \pm SE. (Scale bars, 2 mm.)

Topo VI complex association with the promoter of the *AAA-ATPase* gene and two new loci identified in cluster 4, *At1g24145* and *At1g24147*, the induction of which in the *flu* mutant following a D/L shift is very high and dramatically reduced in *flu caa39* (Dataset S1), making them very suitable for such analysis. *Arabidopsis* transgenic lines that express tagged versions of the AtTOP6A/AtSPO11-3/RHL2/BIN5 (HA-RHL2) and RHL1/HYP7 (RHL1-CFP) Topo VI subunits (25) were crossed with mutant plants deficient for the corresponding genes (i.e., *rhl2-1* and *rhl1-2*) to control functionality of the fusion proteins by phenotypic and molecular complementation. HA-RHL2 and RHL1-CFP restored a wild-type phenotype in *rhl2-1* and *rhl1-2*, respectively, as well as a near wild-type level of transcripts (Fig. S5). ChIP-PCR experiments with the double homozygous lines *rhl2-1 HA-RHL2* and *rhl1-2 RHL1-CFP* revealed that *AAA-*

ATPase sequences were enriched in precipitated chromatin from *rhl1-2 RHL1-CFP* and, to a lesser extent, *rhl2-1 HA-RHL2* (Fig. 5). Enrichment was even more pronounced for *At1g24145* and *At1g24147* sequences under high-light stress conditions that activate the genes (Fig. 5 and Fig. S6). In contrast, no enrichment was observed in the pseudogene *At4g03760* that was used as a control. These results suggest that the plant Topo VI complex directly binds to the promoter/transcription initiation site of *AAA-ATPase*, *At1g24145*, and *At1g24147* genes and, hence, appears to be directly involved in initiation and elongation of $^1\text{O}_2$ -responsive gene transcription.

Discussion

Topo VI, A Genuine Component of $^1\text{O}_2$ Retrograde Signaling. The *caa39* mutant was isolated in a genetic screen aimed at identifying factors involved in $^1\text{O}_2$ -specific retrograde signaling from the plastid to the nucleus and was shown in the present work to be impaired in the Topo VI A-subunit. Initially, six *caa* mutants were isolated, which constitutively activate the *AAA-ATPase* gene in the absence of enhanced $^1\text{O}_2$ production (14). The characterization of these mutants suggested that $^1\text{O}_2$ -signaling does not operate as an isolated linear pathway but rather forms an integral part of a signaling network that is modified by other signaling routes and impacts not only on stress responses of plants, but also on their development. The work suggested further that most of the factors mutated in *caa* mutants repress the basal expression of the *AAA-ATPase* gene but are unlikely directly linked to $^1\text{O}_2$ -signaling. *caa39* was the only *caa* mutant that showed an impaired $^1\text{O}_2$ -induced accumulation of *AAA-ATPase* transcripts and other $^1\text{O}_2$ -responsive genes, and hence AtTOP6A seems to form a genuine part of $^1\text{O}_2$ signaling. The regulatory role of AtTOP6A depends on its function as a subunit of the plant Topo VI complex. As shown by our ChIP analysis, the Topo VI directly interacts with the upstream region of the *AAA-ATPase*, *At1g24145*, and *At1g24147* genes under high-light stress conditions that activate the genes; therefore, we propose that Topo VI is directly involved in initiation and elongation of the transcription of these and other $^1\text{O}_2$ -responsive genes. Nevertheless, a fairly large proportion of nuclear genes activated in the *flu* mutant following a D/L shift are unaffected by the *caa39* mutation. Because plastid-generated $^1\text{O}_2$ is unlikely to leave the chloroplast in *Arabidopsis* (13), this observation supports that several $^1\text{O}_2$ -derived signals might be

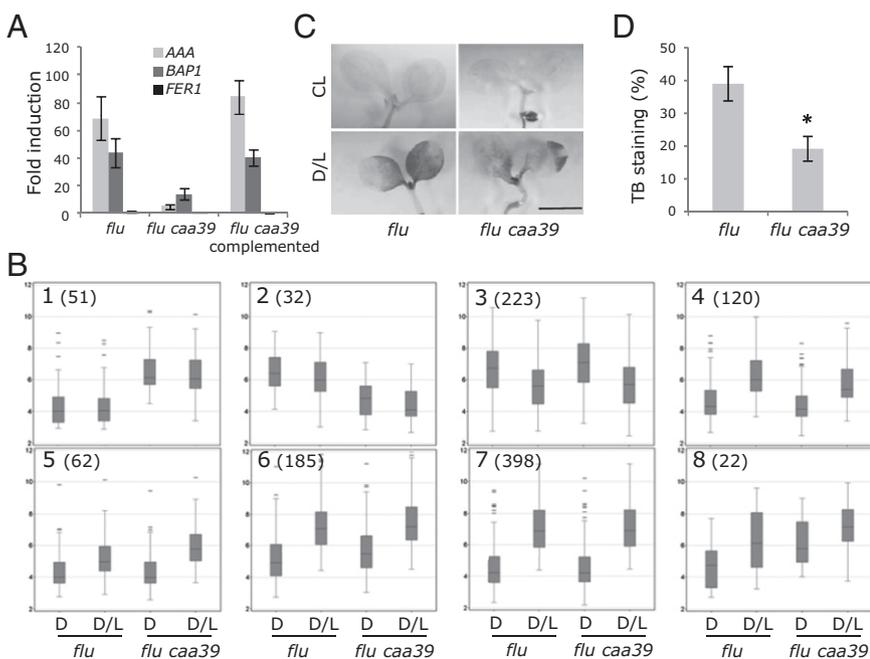


Fig. 3. Response of the *flu caa39* mutant to the release of singlet oxygen. (A) Fold-inductions of *AAA-ATPase*, *BAP1*, and *FER1* in *flu*, *flu caa39*, and *flu caa39* complemented with the wild-type copy of the *AtTOP6A* gene. The 5-d-old seedlings were subjected to 8 h dark and 30 min reillumination (D/L). Transcript levels were determined by quantitative RT-PCR. Results represent mean values of two biological replicates \pm SE (B) Genome-wide identification of 1,093 genes differently regulated in *flu* or *flu caa39* prior or after the release of $^1\text{O}_2$. K-means analysis was used to define eight clusters identifying different expression profiles. (C) Trypan blue staining showing cell death in continuous light (CL) and after D/L shift in 5-d-old seedlings. Plants were subjected to 8-h dark. Trypan blue staining was done after 12 h of light exposure. (Scale bar, 2 mm.) (D) Quantification of cell death. Values are expressed as percentage of positive Trypan blue staining area relative to total area ($n = 8$; mean \pm SE). * t test $P < 0.01$.

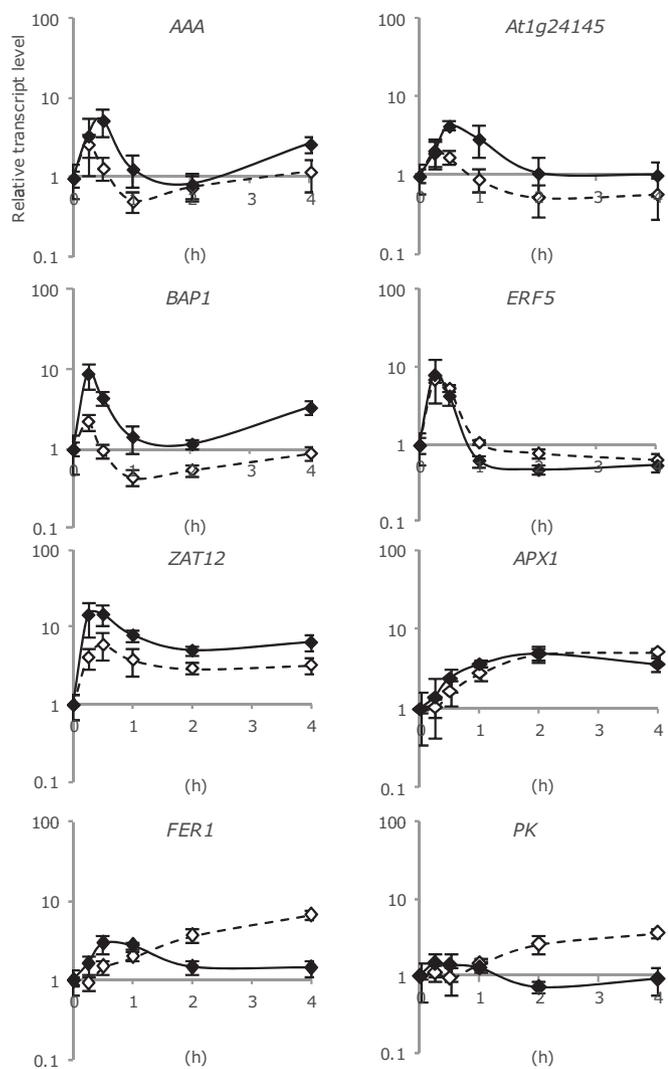


Fig. 4. Response of the wild-type Col and *caa39* mutant to high-light stress conditions. Relative transcript levels of the $^1\text{O}_2$ -responsive AAA-ATPase, *At1g24145*, *BAP1*, *ERF5*, the H_2O_2 -responsive *FER1*, *PK*, and *APX1* genes, and the general ROS-responsive marker gene *ZAT12* were analyzed in wild-type Col (closed symbols) and *caa39* (open symbols) seedlings at the onset and 15 min, 30 min, 1 h, 2 h, and 4 h after the initiation of the high light (HL, $2,000 \pm 50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) treatment. Before HL stress, seedlings were grown for 5 d in normal light (NL, $80 \pm 5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) 16-h light/8-h dark photoperiod conditions. Relative transcript levels were determined by quantitative RT-PCR and expressed relative to the levels in NL. Results represent mean quantification cycle values of four biological replicates \pm SE.

transferred from the plastid to the nucleus and modify the activity of Topo VI—or Topo VI-dependent nuclear factors—as well as of other still unknown nuclear factors that control the expression of Topo VI-independent $^1\text{O}_2$ -responsive genes. A candidate $^1\text{O}_2$ -derived signal could be β -cyclocitral, a carotenoid oxidation product that was recently found to accumulate under high light conditions that generate $^1\text{O}_2$ (30). However, because β -cyclocitral-mediated signaling does not appear to require the EXECUTER1 function, it seems to be different from the EXECUTER1-dependent $^1\text{O}_2$ -signaling pathway operating in the *flu* mutant subjected to a D/L shift and wild-type plants exposed to mild light stress (31).

Topo VI, An Integrator of Different ROS Signals. Our findings show that the plant Topo VI complex represses the expression of AAA-ATPase under nonstress conditions but can act as an activator of the AAA-ATPase and other genes in response to $^1\text{O}_2$. At

the same time, the identification of genes that were further activated in *flu caa39* following a D/L shift supports a role of Topo VI in both activation and repression of $^1\text{O}_2$ -regulated genes. Furthermore, under ROS-producing high-light stress conditions, Topo VI can work simultaneously as an activator and a repressor of different ROS-responsive genes (i.e., $^1\text{O}_2$ -responsive vs. H_2O_2 -responsive genes), suggesting that it might act as a molecular switch that relays the known antagonistic effect of H_2O_2 to $^1\text{O}_2$ -signaling (11). This dual activity assigns a key role to Topo VI as an integrator of different ROS signals that are released by plants in response to adverse environmental conditions. Other type II topoisomerases have also been shown to act as transcriptional repressors (32). The human topoisomerase II (hTopo II) represses RNA polymerase II transcription in vitro by binding to the promoter and blocking the formation of stable preinitiation complexes. Significantly, hTopo II-mediated repression can be relieved by the addition of sequence-specific transcriptional activators (32, 33). In addition, a dual role for human DNA topoisomerase I (hTopo I) as transcriptional repressor and activator has been reported (34). It is proposed that hTopo I is loaded onto the transcription complex to repress transcription by interacting with the transcription factor II D (TFIID) complex. In the presence of an activator, hTopo I is assumed to then be translocated from the TFIID complex to the elongation complex, thereby removing the superhelical tension caused by the elongation process and enhancing the efficiency of elongation (34). The dual activity of *Arabidopsis* Topo VI as a repressor and activator of $^1\text{O}_2$ -responsive gene expression could be explained by an analogous mechanism. This hypothesis is further supported by the recent finding that the *Arabidopsis* BIN4/MID subunit of Topo VI can interact in a yeast two-hybrid assay with the TFIIB, which is involved in RNA polymerase II recruitment and transcription initiation in eukaryotes (35, 36). Quite recently, Ju et al. provided in vivo molecular evidence that human Topo II β can generate a transient dsDNA strand break that permits a nucleosome-specific histone H1–HMGB protein exchange event, which promotes local changes of chromatin architecture and leads to the transcriptional activation of target genes (37). Topoisomerase-mediated chromatin factor exchange represents an attractive mechanism for the transcriptional activation and repression of different sets of genes. The mechanism of *Arabidopsis* Topo VI activation following the release of different ROS remains to be elucidated, as does the identification of other determinants that may control the selectivity of response to different ROS.

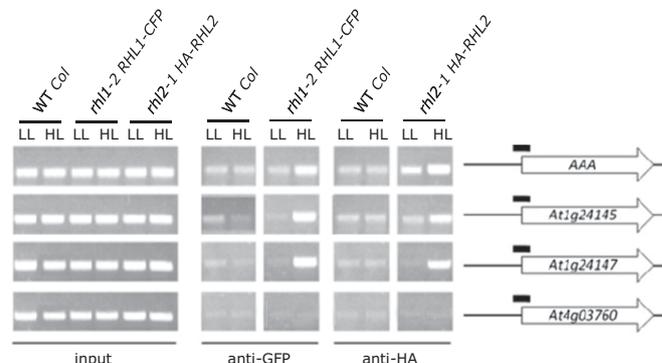


Fig. 5. Identification of Topo VI binding sites in vivo. ChIP assays were carried out using anti-GFP and anti-HA antibodies with wild-type (Col), *rhl1-2 RHL1-CFP*, and *rhl2-1 HA-RHL2* plants that were grown for 6 d in low light (LL; $12 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and then transferred to moderate high light (HL; $1,050 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 30 min. PCR analysis was carried out to probe RHL1 and RHL2 binding to the promoter region of the $^1\text{O}_2$ -responsive AAA-ATPase, *At1g24145*, and *At1g24147* genes and the control locus *At4g03760*. Filled boxes indicate position and size of PCR-amplified fragments. The experiment was repeated three times with comparable results.

Topo VI, An Integrator of Environmental Cues. Until now, Topo VI was primarily implicated in DNA endoreduplication, and the physiological significance of plant topoisomerases was largely unknown (17, 23–25). Interestingly, recent studies have shown that the constitutive expression of rice *OsTOP6A* or *OsTOP6B* increases the expression of stress-responsive genes and confers abiotic stress tolerance to transgenic *Arabidopsis* plants (38, 39). Our study shows that the plant Topo VI complex is a key regulatory factor during the activation of ROS-responsive genes that can eventually modulate the intensity of the $^1\text{O}_2$ -induced cell-death response. The *Arabidopsis* genome contains a second type II topoisomerase that is, however, differentially regulated (23, 40) and, based on our preliminary data, doesn't seem to participate in ROS-responsive gene activation. Taken together, these findings suggest that Topo VI may have a specific function in regulating plant responses to adverse environmental conditions by reprogramming the expression of specific sets of ROS-responsive genes.

Materials and Methods

Plant Material and Growth Conditions. All experiments were performed with *A. thaliana* ecotype *Columbia* (Col-0). Details of lines and growth conditions are given in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Plant Material and Growth Conditions. All experiments were performed with *Arabidopsis thaliana* ecotype *Columbia* (Col-0). The *flu* Col-0 line used in this work had been obtained by five backcrosses of *flu1-1* (1) in *Ler* with wild-type Col-0. Other lines used in this study were: *flu* AAA:*LUC*⁺ (2); *flu* AAA:*LUC*⁺ *caa39*, *flu* *caa39*, obtained by a backcross of *flu* AAA:*LUC*⁺ *caa39* to *flu* Col-0 and *caa39* obtained by backcrosses to wild-type Col-0; *spo11-1-3* (SALK_146172); *spo11-2* (SAIL_551_F05); *bin5*, kindly provided by J. Chory (The Salk Institute, San Diego); *rhl2-1*, *hyp6*, *rhl1-2*, and *bin4-1*, kindly provided by K. Sugimoto-Shirasu (Riken Institute, Yokohama, Japan); and *RHL1-CFP* and *HA-RHL2* transgenic lines, kindly provided by Viktor Kirik (Carnegie Institution of Washington, Pasadena, CA). Sequences of primers used for genotyping the mutant lines are listed in Table S5. Seeds were surface-sterilized and grown on Murashige and Skoog (MS) medium (without sucrose) including vitamins and 2-(N-morpholino)ethanesulfonic acid (MES) buffer (M0255; Duchefa) and 0.8% (wt/vol) agar (Sigma-Aldrich) at 20 °C in continuous light (80–100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) unless otherwise indicated. High-light stress experiments were performed using FYTO-LED light panels (SL3500; Photon Systems Instruments).

Identification and Complementation of the *caa39* Mutation. A segregating F2 mapping population was generated from a cross of *flu* AAA:*LUC*⁺ *caa39* in Col-0 with *flu* AAA:*LUC*⁺ in *Ler*. Of 1,700 F2 plants, 500 homozygous *flu* AAA:*LUC*⁺ *caa39* mutants were selected based on high constitutive luciferase expression in continuous light. The *CAA39* locus was mapped using CAPS (cleaved amplified polymorphic sequence) or SLP (simple sequence length

polymorphism) markers listed in The *Arabidopsis* Information Resource database (TAIR, www.arabidopsis.org). Additional markers used for mapping were designed based on the collection of predicted *Arabidopsis* SNP and small insertions/deletions in the publicly available *Columbia* and *Landsberg erecta* sequences generated by Monsanto (<http://www.arabidopsis.org/Cereon>) and are provided in Table S1. For complementation, the full-length coding sequence of *AtTOP6A* amplified by PCR (Table S2) was cloned in pCAMBIA1302 binary vector under the control of the CaMV 35S promoter and introduced into *flu* *caa39* via *Agrobacterium*-mediated transformation as described (3). Positive transformants were selected on hygromycin-containing media.

RNA Isolation, Quantitative RT-PCR, and Microarray Analysis. Quantitative RT-PCR was performed as described previously (2). Sequences and efficiencies of primers used for qRT-PCR are listed in Table S3. Validation of the reference genes *PRF1*, *ACT2*, *PP2AA3*, *GAPC2*, and *UPL7* (4) was performed with geNorm (5) and is presented in Fig. S7. Microarray experiments were performed with three biological replicates, and full-genome Affymetrix *Arabidopsis* AGRONOMICS1 microarrays (Affymetrix) were used. Labeling of samples, hybridizations, and measurements were performed as previously described (6, 7). Signal values were derived using the RMA algorithm implemented in the statistical language R (8). Differentially expressed genes were selected using LIMMA (9) followed by multiple testing correction according to ref. 10. Genes were considered as differentially expressed if $P < 0.05$ and fold-change at least 1.5. The microarray data have been submitted to ArrayExpress with the experiment number E-TABM-1076.

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Sulfolobus      : -----MSSEFISKVDKEARRKAA : 18
Pyrococcus     : -----MKLKRKPKKFKFSYDPQKWL : 20
Methanosarcina : -----MEGEKGSKTRKGDALAR : 17
Arabidopsis    : MAD-----KKKRKRKSDDEAEELPFKSILESDDVITELLKSYISSSIKAAAGAGASSSSSKPLTLADLSLSSSCREVADLSLSS : 82
Populus        : MADS-----TSTKSRKRQPPDPSTTELLFKNLLKPSVILQTLQSLVIS-----T--ASASSSKPLTSLDLSLSSSCREVADLSLSS : 78
Oryza          : MSEKRRRGAGAGAASGSASKPRVTAASYAESLRSLKRPDASILATLRLSASCSKSPAGSSSSSSSASKALAAEDDPAASYIVVADQDSASVT : 97
Physcomitrella : MSS-----DGPRKQTQAALAFKKKLSKDPDILKIVTKFAEA-----AKEADVETRLADLNLANSCKEVEVNLNTRSV : 68
Chlamydomonas : MSK-----RGGADDKALALAKGKRIRGAEDVLKKVQALATQ-----LKNKSEVKTLLAEDLDPYNCREVVDDKGGDDM : 68
Ostreococcus  : MSAB-----RRKVSADAGVNFALARLRDGEDGRPGSTSEVLRAVN-----DLEREVKARGVKRKTLDLSIAEAYREVDVGGEEA : 77
Thalassiosira : -----MNTTQM : 7
Phaeodactylum : MASRNRREPLAS----GPAPRPDMSATSTRKRKAVALAADLVLKRCRQLRQSLKAEK-----AEIESSVVGVLPPEGIPSDIVEVLPATQWL : 85
Cyanidioschyzon : MVAKRKTKATEPR---LSTASRKKWQAESGAVSSGTAADVAESFLNRVRELKREIESRE-----AAKQRFILQSSDASSAARIEVELDCDQWL : 88
Bdellovibrio  : -----MAKLLSIRDLKIDIP : 15
    
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rhl-2

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Sulfolobus      : NIIRDKFLINVEQIKKGEPLVMEIEMTLNANLYDEKRLKLLIGEKLRNENLDINEAKFMQIVLMASTIY-DALVSDPEYFPIRDIYRGRKSHLL : 114
Pyrococcus     : KKLEDAAYKILEEVKAGKNPYFDVPTRGLSNVYFDEEARLKLKGDRLSRRYELNVAHAKRFMQLIMAYVK-RIVSEGKIASLREAYANKHTIPG : 116
Methanosarcina : EKLEIAEKIYNQFEEVVPVSVLPSPTKANLAYSDESDVWVYGDRESESAKTVKGAQQLLKYATYDFLINEHLARNGRSTLRELYYISEGWDYA : 114
Arabidopsis    : TELLETIVICLARSLLAGDGFSEFVPSRAASNOLYVPELDRIVKDRSTLPEASVSSVRKTTITRILLAIH-QLCLRNIHYTKRDLFYDVKLFQD : 178
Populus        : SELEAATISIVQSLILSGKGFSEFVPSRAASNOLYVPELDRIVKDRNTLPEANLSSVRKCTITARLISLIH-QLCLRNIHYTKRDLFYDVKLFQD : 174
Oryza          : SSINRVLAAARSILSGRGFSEFVPSRAASNOYVLPDLNRIIVVRESANPQANVATARKATTIARVLFVH-AVLRRIHIVTKRDLFYDVKLFQD : 193
Physcomitrella : LEVKSILAKSILSGNGFGYLLPSRSSANOLYVPELDRIVKDRASFPQANASVRRKTAITRRLQVH-QLCTRQIHYTKRDLFYDVKLFQD : 164
Chlamydomonas : GELAAAMYNVAASILGGEGFAFVPSRAAGNOLYVPELDRIVKDRVAVSKPEASTATCRKAAVITRLLGVH-ELLGKNIHYTKRDLFYDVKLFQD : 164
Ostreococcus  : AALEQVMIAVARSVMRGGDFEYTPSRGATDQVYVEALDRIVKNTTRAAEAGTVSSVRKVTILTRVMOVH-VGLSEGIHYTKRDLFYDVKLFQD : 173
Thalassiosira : EGENAVKIAKQVLAKQGFQLEIPSRASNOYVPELDRIVKDRGTGTSEFLNVEKSRKSAITRRLQVH-AVLLKRIHYTKRDLFYDVKLFQD : 103
Phaeodactylum : EGVEAALHIAQVLERKGFSEIPSRASNOLYVPELDRIVKDRVAVSKPEASTATCRKAAVITRLLGVH-ELLGKNIHYTKRDLFYDVKLFQD : 181
Cyanidioschyzon : AELERVILSAKSLLEGRGLSYKVPRESAGNOYVPELELERVVKDVAISN-ASVAVSRKTTIMTRVQVH-ELCTRRIHYTKRDLFYDVKLFQD : 184
Bdellovibrio  : KEARIADKVLKDESSKRPVLEAVKTSLDMSLYNAKVGYLTPGDIVVTELVNVSVOELARVVELEMLL-RNLETGTVNFKRELYYMCKGEIKG : 110
    
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CAP

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Sulfolobus      : KSIEGNKIVSEENTWDECKFSDSVIVTEVFTSLREEMLIISKEKGGK-VGENTRIRSGN-----DVIDLSKTHGAYAEETPDLIDF-DVDAE : 203
Pyrococcus     : -----T-HENTFEDCSFSDPIEBLERMLQVLRREEMHITDRRGY-VYGDIVIKDGE-----DEFNASKLGGGAVVGTVEHIQF-PEINVD : 196
Methanosarcina : -----KFKCCESDRLIELEITLSLOREYFHMPEEDGATVFGPIETETQTKR-GERNIHCQKVDGEGGYQIFNVENIEF-QKHDS : 196
Arabidopsis    : QTQSDAVLDDVSCMLGCTRSSNLVIAAEKGV-VVGRLIIFSDNG-----DMIDCTKMGVGGKAI-PNIDRVGD-VQSDAM : 250
Populus        : QTQSDAVLDDVSCMLGCTRSSNLVIAAEKGV-VVGRLIIFSDNG-----DMIDCTKMGVGGKAI-PNIDRVGD-VQSDAL : 246
Oryza          : QAQSDAVLDDVSCMLGCTRSSNLVIAAEKGV-VVGRLIIFSDNG-----DRIDCTKMGVGGKAI-PNIDRVSG-VESDAL : 265
Physcomitrella : CGQSDAILDDVSCMLGCTRSSNLVIAAEKGV-VVGRLIIFSDNG-----DRIDCTKMGVGGKAI-PNIDRVGD-VESDAL : 236
Chlamydomonas : QSQSDAILDDVSCMLGCTRSSNLVIAAEKGV-VVGRLIIFSDNG-----DFIDCTKMGVGGKAI-PNIDRVGD-VESDAL : 236
Ostreococcus  : QKQSDAVLDDVSCMLGCTRSSNLVIAAEKGV-VVGRLIIFSDNG-----DEIDCTKMGVGGKAI-PNIDRVGD-VESDAL : 245
Thalassiosira : CSQSDAVLDDVSCMLGCTRSSNLVIAAEKGV-VVGRLIIFSDNG-----DFIDCTKMGVGGKAI-PNIDRVGD-VESDAL : 175
Phaeodactylum : QAQSDAVLDDVSCMLGCTRSSNLVIAAEKGV-VVGRLIIFSDNG-----DFIDCTKMGVGGKAI-PNIDRVGD-VESDAL : 253
Cyanidioschyzon : QTQSDAVLDDVSCMLGCTRSSNLVIAAEKGV-VVGRLIIFSDNG-----DFIDCTKMGVGGKAI-PNIDRVGD-VESDAL : 256
Bdellovibrio  : NP-----RLKPLDFDDCPESDALIFIGDMIEVYREELNVFANDRGGQYSQQLVVTETLDGDKAVILDLSTLSTSPFPQKPKQALKLLKAKKID : 201
    
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caa39

bin5

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Sulfolobus      : FVLVVEKDAVMEQOLHHRAGFKQIKSLLISAGOPDRAIRREVRRLNEELKLPVYITDADBYGWIYFVSHRI-GSISLSVESERTATPDAKFTGVMS : 299
Pyrococcus     : YVLMVEETAMADRLIEKYPKREKRALIATQCGASRGVRRILRILHYEGLPLIIVTDDGDFYGYIYSTIKQ-GSINILANLEKATPAKFGMTM : 292
Methanosarcina : MIAIATPGGMVYARLMEGDEANAILVHLKQGPARTRIIKRMNEEIGPVAVETDGDPEWSYR-YASVAG-CALKSAHLSEFVAAPAKFTGLQF : 292
Arabidopsis    : FILLVEKDAAYMRLAEDRFVNRFPCCIIVTAKGQPDVATRFLLRKKMTELKLPVALVDSDFYGLKILSVYGC-GSKNMSYDSANLTPDIKWLGVRP : 346
Populus        : FILLVEKDAAYMRLAEDRFVNRFPCCIIVTAKGQPDVATRFLLRKKMTELKLPVALVDSDFYGLKILSVYGC-GSKNMSYDSANLTPDIKWLGVRP : 342
Oryza          : FILLVEKDAAYMRLAEDRFVNRFPCCIIVTAKGQPDVATRFLLRKKMTELKLPVALVDSDFYGLKILSVYMC-GSKNMSYDSANLTPDIKWLGVRP : 361
Physcomitrella : FILLVEKDAAYMRLAEDRFVNRFPCCIIVTAKGQPDVATRFLLRKKMTELKLPVALVDSDFYGLKILSVYMS-GSKNMSYDSANLTPDIKWLGVRP : 332
Chlamydomonas : FILLVEKDAAYMRLAEDRFVNRFPCCIIVTAKGQPDVATRFLLRKKMTELKLPVALVDSDFYGLKILSVFVLSGSMNMSYDSANLTPDIKWLGVRP : 333
Ostreococcus  : FILLVEKDAAYMRLAEDRFVNRFPCCIIVTAKGQPDVATRFLLRKKMTELKLPVALVDSDFYGLKILSVYMC-GSKNMSYDSANLTPDIKWLGVRP : 341
Thalassiosira : FILLVEKDAAYMRLAEDRFVNRFPCCIIVTAKGQPDVATRFLLRKKMTELKLPVALVDSDFYGLKILSVYMS-GSKNMSYDSANLTPDIKWLGVRP : 271
Phaeodactylum : FILLVEKDAAYMRLAEDRFVNRFPCCIIVTAKGQPDVATRFLLRKKMTELKLPVALVDSDFYGLKILSVYMS-GSKNMSYDSANLTPDIKWLGVRP : 349
Cyanidioschyzon : FILLVEKDAAYMRLAEDRFVNRFPCCIIVTAKGQPDVATRFLLRKKMTELKLPVALVDSDFYGLKILSVYMS-GSKNMSYDSANLTPDIKWLGVRP : 352
Bdellovibrio  : FCLVVESEGANTLVTMGFTKRNNQIVMGAQGVESVNGVGRKLIQEBIDVPMYFFGDADAYTMONIFRTLKAGSAAASLIRNADFSPANNVKEFGVLP : 298
    
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TOPRIM

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Sulfolobus      : GDIFGSRKPKPYLSEAERKNYIKAKDADIKRAEIKNYEWFK---TKAQBEINTFQORAKLETEAMASKG---LKFIAFOYIPEKITTNKDYTA : 389
Pyrococcus     : DDIKENYGLHEVTEKLGIPDPKGGPTGYKRLIEENYFPWFQ---NKEWOROLKIALKGVVRIEQQALANKS---LEFVAKBYLPEKTRFGKLLP : 382
Methanosarcina : SDIVEVEIS-----TDKLEQDYSALRSEISDRPFE---SDYKKBQIQHQLDIGKKAQQQFAGK---LDFVTEVYLLRNREKFMGMT : 369
Arabidopsis    : SLDLKYKIF-----EQCRIPMTEQDIKTGKMLLEEDFVK---NPGWVEELSLMVKTRKAKAETQALSSFG---FOYLSBYLLKLLKQQDWT : 427
Populus        : SLDLKYKIF-----EQCRIPMTEQDIKTGKMLLEEDFVK---NPGWVEELSLMVKTRKAKAETQALSSFG---FOYLSBYLLKLLKQQDWT : 423
Oryza          : SLDLKYKIF-----EQCRIPMTEQDIKTGKMLLEEDFVK---NPGWVEELSLMVKTRKAKAETQALSSFG---FOYLSBYLLKLLKQQDWT : 442
Physcomitrella : SLDLKYKIF-----EQCRIPMTEQDIKTGKMLLEEDFVK---NPGWVEELSLMVKTRKAKAETQALSSFG---FOYLSBYLLKLLKQQDWT : 413
Chlamydomonas : SLDLKYKIF-----EQCRIPMTEQDIKTGKMLLEEDFVK---NPGWVEELSLMVKTRKAKAETQALSSFG---FOYLSBYLLKLLKQQDWT : 414
Ostreococcus  : SLDLKYKIF-----EQCRIPMTEQDIKTGKMLLEEDFVK---NPGWVEELSLMVKTRKAKAETQALSSFG---FOYLSBYLLKLLKQQDWT : 422
Thalassiosira : SLDLKYKIF-----EQCRIPMTEQDIKTGKMLLEEDFVK---NPGWVEELSLMVKTRKAKAETQALSSFG---FOYLSBYLLKLLKQQDWT : 352
Phaeodactylum : SLDLKYKIF-----EQCRIPMTEQDIKTGKMLLEEDFVK---NPGWVEELSLMVKTRKAKAETQALSSFG---FOYLSBYLLKLLKQQDWT : 430
Cyanidioschyzon : TDLDRYGLF-----EQCRIPMTEQDIKTGKMLLEEDFVK---NPGWVEELSLMVKTRKAKAETQALSSFG---FOYLSBYLLKLLKQQDWT : 433
Bdellovibrio  : EDVKKYDLF-----HYKVESDPAEARALKKAKDALENDPFLDKKNKLNADILRLWIKERTRCBQCSFFSVDPKDPKTKLILEKIKRGSYV : 387
    
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Fig. S2. Multiple alignment of TOP6A/SPO11-3 proteins from archae, plants, green algae, diatoms, red algae, and proteobacteria. The proteins were aligned using CLUSTALW. Conserved CAP and topoisomerase-primase (TOPRIM) domains are indicated. Arrows show position of the amino acid exchange caused by *caa39* mutation and positions of *rhl-2* and *bin5* mutations. Accession numbers are as follows: *Sulfolobus shibatae* CAA71605.1; *Pyrococcus furiosus* AAL81702.1; *Methanosarcina mazei* NP_634442.1; *Arabidopsis thaliana* NP_195902.1; *Populus trichocarpa* XP_002326329.1; *Oryza sativa* CAD79468.1; *Physcomitrella patens* XP_001763679.1; *Chlamydomonas reinhardtii* XP_001701722.1; *Ostreococcus tauri* CAL53806.1; *Thalassiosira pseudonana* XP_002294481.1; *Phaeodactylum tricornerutum* XP_002177352.1; *Cyanidioschyzon merolae* CMQ111C; *Bdellovibrio bacteriovorus* CAE80644.1.

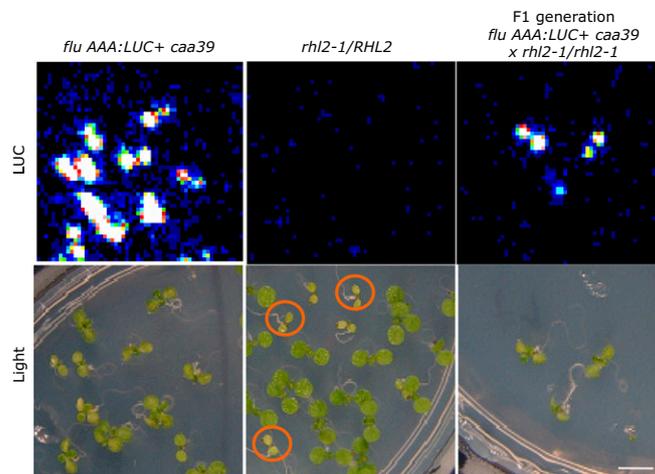


Fig. S3. Allelism test of *flu AAA:LUC⁺ caa39* with *rh12-1* mutant. *flu AAA:LUC⁺ caa39* mutant was crossed with the homozygous *rh12-1* mutant selected based on the mutant phenotype from the segregating *rh12-1/RHL2* F2 generation that was grown under long-day conditions. F1 generation seeds were plated on MS agar plates and grown under constant light (CL) conditions. Luciferase image (LUC) and visible picture (Light) of *flu AAA:LUC⁺ caa39* seedlings, F2 segregating *rh12-1/RHL2* population, and F1 seedlings from the *flu AAA:LUC⁺ caa39* × *rh12-1/rh12-1* cross-over are presented. Orange circles mark homozygous *rh12-1* plants. (Scale bar, 0.5 cm.)

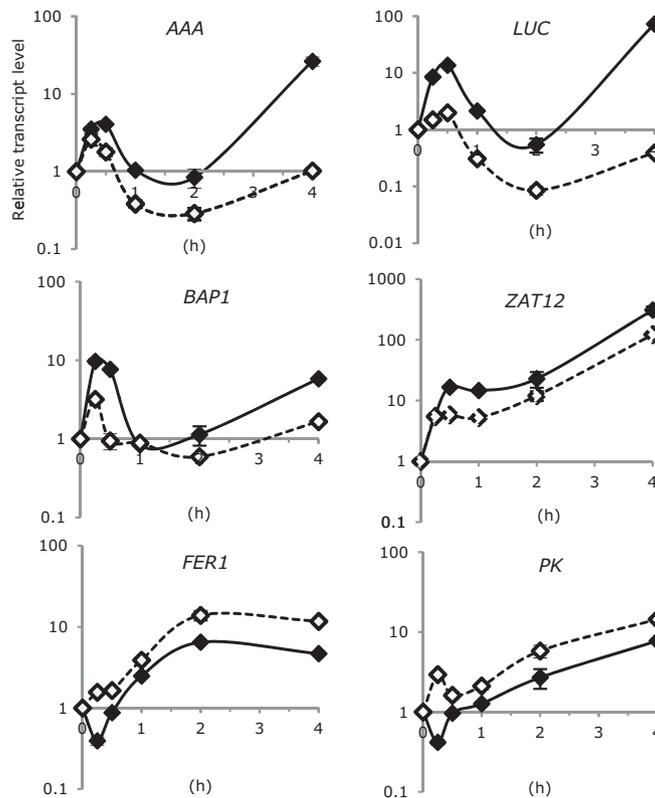


Fig. S4. Response of the *flu AAA:LUC⁺ caa39* mutant to high light stress conditions. Relative transcript levels of the $^1\text{O}_2$ -responsive *AAA-ATPase*, *LUC* and *BAP1* genes, the H_2O_2 -responsive *FER1* and *PK* genes and the general reactive oxygen species (ROS)-responsive marker gene *ZAT12* were analyzed in *flu AAA:LUC⁺ caa39* (closed symbols) and *flu AAA:LUC⁺ caa39* (open symbols) seedlings at the onset and 15 min, 30 min, 1 h, 2 h, and 4 h after the initiation of the high light (HL, $1,050 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) treatment. Before HL stress, seedlings were grown for 6 d in low light (LL, $12 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Relative transcript levels were determined by quantitative RT-PCR and expressed relative to the levels in LL. Results represent mean values of two technical replicates \pm SE.

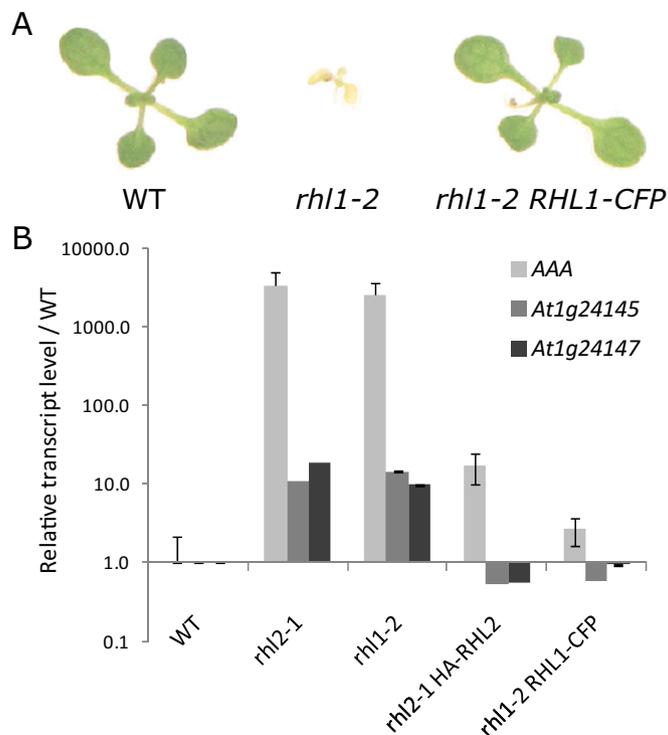


Fig. S5. Functional complementation of *rh1-2* and *rh1-1* by HA-RHL2 and RHL1-CFP respectively. (A) Morphological phenotype of wild-type, homozygous *rh1-2*, and double homozygous *rh1-2/rh1-1* RHL1-CFP/RHL1-CFP 10-d-old plants. Similar complementation of the morphological phenotype of the *rh1-2* homozygous mutant was obtained with HA-RHL2. (B) HA-RHL2 and RHL1-CFP restored a near wild-type level of transcripts in *rh1-2* and *rh1-1*, respectively. Results represent mean values of two biological replicates \pm SE.

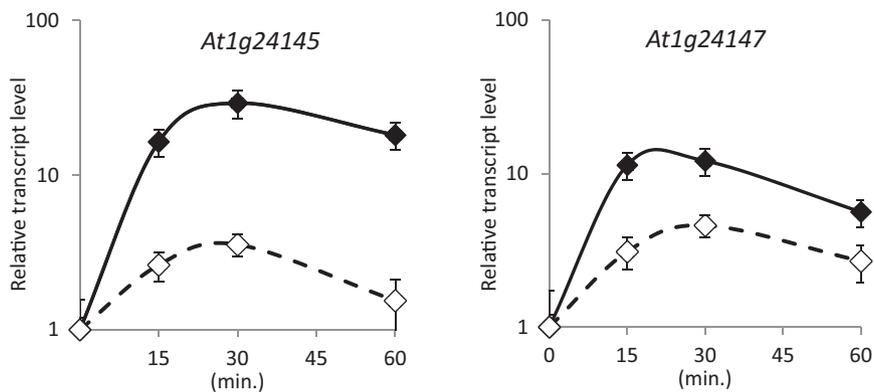


Fig. S6. Activation of the *At1g24145* and *At1g24147* genes in response to high light stress in wild-type (closed symbols) and *caa39* (open symbols). Relative transcript levels were analyzed in seedlings that were grown for 6 d in low light (LL, $12 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and then transferred to moderate high light (HL, $1,050 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 15 min, 30 min, and 1 h. Relative transcript levels were determined by quantitative RT-PCR and expressed relative to the levels in LL. Results represent mean values of two biological replicates \pm SE.

Table S1. Polymorphic markers used for rough and fine mapping of the *caa39* mutation

chromosome or name	Position (bp)	Name	Forward primer 5' to 3'	Reverse primer 5' to 3'
Polymorphic markers used for rough mapping of the <i>caa39</i> mutation				
I	3164003	F21M12	GGCTTTCTCGAAATCTGTCC	TTACTTTTTGCCTCTTGTCATTG
	9621344	CIW12	AGGTTTTATTGCTTTTCACA	CTTTCAAAGCACATCACA
	20877364	NGA280	GGCTCCATAAAAAGTGACCC	CTGATCTCACGGACAATAGTGC
	28185746	ATPase	GTTACAGAGAGACTCATAAACCA	CTGGGAACGGTTCGATTCGAGC
II	1078851	F19B11	CAAAGCTTTTGCTCTTGC	TGGGTTTTGATGTTTTCTTT
	13875000	T21L14	TTCAAAGTTTCCACCTCATGC	TATGTGTGAGGCCAAGAACC
	16885036	T3G21	CTCGTTCGTGCGTAGC	AAGCCAAGCAAACGCATC
III	178000	F4P13	CAAGCCAAAAGGTCTTCACC	TAATCCAGCCTCGCAAC
	9775545	CIW11	CCCCGAGTTGAGGTATT	GAAGAAATTCCTAAAGCATT
	18901818	CIW4	GTTCAATAAACTGCGTGTGT	TACGGTCAGATTGAGTGATTC
	22309429	T8B10	AGTGCTATTTGTAATCGCT	TCAACATTTCATTTTTCCA
IV	747854	CIW5	GGTAAAAATTAGGGTTACGA	AGATTTACGTGGAAGCAAT
	8110273	FCA6	CATGTTATCAGGACAATCTAACG	GCTTCGGCACCAGACTTGTA
	1164575	T10P11	TCATGATAACACAGGGCGTAA	TTCCGATATCTCATGCCATC
	1617020	ATA22	CTTTAGATGCTATACTAGGTT	CGATTTTGCATTGTTTTTATG
V	980000	MED24	TGCCTCCTGGGAAAGTG	GGCCCAAGCACACCTACA
	7428725	MWD9	CTCTACTAAATTTTCAGCT	TACGAATGTATTCACATGCA
	14952821	MPA22	GGTCTTGAATCGCAGCATA	GATGATAGTGACACCTTCTCATGT
	19493000	K20J1	TGGGAAGACGATGATGGA	CCGCATGATGCATAGCAA
Polymorphic markers used for fine mapping of the <i>caa39</i> mutation				
T20L15	340947	SSLP	AGATTGTTTCTCTCAGTGCTC	AGATTGTTTCTCTCAGTGCTC
T7H20	436559	SSLP	GAGGAGTTGTGCAAGTTGAG	TTAGTACCTAGCTAACGGACCT
T22P11	586645	CAPS	AGGCGTAACCATTGCACAC	ACGACGATCAGAGAACTAAGC
F9G14-1	620980	CAPS	TAAGAGGAACATAAGAAGCCTG	CCCATATTCTATAGTCAATCGG
F9G14	660584	SSLP	ACAAATTCAT AAGTTTCGTC ACT	TATGCCGTTTACTGATAATCC
F9G14-2	661303	CAPS	CTCTTGAACAACCTCCGAAC	AATGTTTATCATCTGCGGGATG
F15A17	698509	SSLP	GAGAGGTTTATGACCCAACGAC	CTACAAAGCAAATATTCACGCC
F15A17-1	709015	CAPS	GAACACGAGGTCTTGCCCTTG	TCAGATGAATTAGATCAGGATC
F17C5	967107	SSLP	GTTTACCCTTAAGAACCCGA	TGCCGGTACTAGAATATGTT
MED24	1034836	SSLP	GCTTCTATTGGATGATGAGAC	CTACAAGTCCGATTGATTCCA
NGA225	1507038	SSLP	CAGAGGAGAATCGAATTGGAG	CTCATGATTTGGTGTGGTGC

Table S2. Oligonucleotides used for cloning

Gene name	Forward primer 5' to 3'	Reverse primer 5' to 3'
<i>AT5G02820</i>	GAT <u>GGGCC</u> CTTGACATTCGTCTTAC	CTA <u>CTAGT</u> CTGCCTCCGACTG

The underlined bases indicate the *Apal* and *SpeI* restriction enzyme sites added for cloning purposes.

Table S3. Oligonucleotides used for quantitative RT-PCR

Gene name	AGI	Forward primer 5' to 3'	Reverse primer 5' to 3'	Efficiency (%)
<i>PP2AA3</i>	<i>At1g13320</i>	CAGTATCGTCTCTCGCTCCAG	GTTCTCCACAACCGTTGGTC	95.2
<i>UPL7</i>	<i>At3g53090</i>	CTTCTGGGAGGTGATGAAAGG	CTCCAATAGCAGCCCAAAGAG	96.3
<i>Actin2 (ACT2)</i>	<i>At3g18780</i>	CATTCTTGCTTCCCTCAGCAC	CCCAGCTTTTAAAGCCTTTG	97.0
<i>Profilin 1 (PRF1)</i>	<i>At2g19760</i>	AGAGCGCCAAATTTCTCAG	CCTCCAGGTCCCTTCTTCC	98.1
<i>GAPC2</i>	<i>At1g13440</i>	GGCCATCAAGGAGGAATCTG	CCTGGCATCGAAAATGCTTG	100.0
<i>AAA-ATPase (AAA)</i>	<i>At3g28580</i>	GGCAATCTTCTCGTTTTACCC	GCTCATCGTCTTCCCTCTCTC	108.9
<i>At1g24145</i>	<i>At1g24145</i>	AATGGAACATCGCAAACCTCG	GTTGCAATTTGGAGCCTTG	107.2
<i>AT1G24147</i>	<i>At1g24147</i>	CGCAAACCTCAAAGAGATGATCAC	TCAGGAAGATAAGTGGTGACGA	112.3
<i>BAP1</i>	<i>At3g61190</i>	GGTGATAAGTGTGGGATCGTC	GTCTCTAATCTCGGCCTCCA	96.5
<i>ERF5</i>	<i>At5g47230</i>	TTATGTGACTGGGATTTAACGGG	TCAAACAACGGTCAACTGGG	99.0
<i>FER1</i>	<i>At5g01600</i>	CGTTCACAAAGTGGCCTCAG	CAAATCCGTGGCCTTTGC	91.8
<i>PK</i>	<i>At3g49160</i>	CGGAGTTCCAACACTAGAGCTG	AGCTTCAACGATATTCTTGCTC	99.5
<i>APX1</i>	<i>At1g07890</i>	TGCCTTTTCTCGTGATTACG	CAAAAACAGCCATGACTCTCG	110.2
<i>ZAT12</i>	<i>At5g59820</i>	TGCGAGTCACAAGAAGCCTA	GTGCTCTCCCAAAGCTTGTG	91.4
<i>Luciferase</i>		TTACACGAAATTGCTTCTGGTG	CCTCGGGTGAATCAGAATAGC	

Table S4. Oligonucleotides used for CHIP-PCR

Locus name	AGI	Forward primer 5' to 3'	Reverse primer 5' to 3'
Pseudogene	<i>At4g03760</i>	GAAGCGAGACTTTCTGCTCGG	CCGAGGCGGTTGTTGTGCTAC
<i>At1g24145</i>	<i>At1g24145</i>	GATCCCATTTGACCGAGTAAAC	TACCGCATCTTAGAAGTGAATG
<i>At1g24147</i>	<i>At1g24147</i>	TAAATGCGTAAACGTGAGTCGG	CATGAAGTGAAAACACGAGGAC
<i>AAA-ATPase</i>	<i>At3g28580</i>	TTGTGTACCAGAACCACCATC	GGCTTAGGGCTTTGGAAGAG

Table S5. Oligonucleotides used for genotyping

Description	Forward primer 5' to 3'	Reverse primer 5' to 3'
AtSPO11-1-3 WT	TTTCAGTGTAGTCGGTACAACCTGAATGTG	CCACAACCAGTATGTACTCAGCTAAGCTAAC
AtSPO11-1-3 T-DNA	TTTCAGTGTAGTCGGTACAACCTGAATGTG	GCGTGGACCGCTTGCTGCAACT
AtSPO11-2 WT	GGGACTTGAAGCATACAGATACGGTAAAG	CTCGAGTTATATGTATTTGCCTTGACGATCTTGG
AtSPO11-2 T-DNA	GGGACTTGAAGCATACAGATACGGTAAAG	TAGCATCTGAATTCATAACCAATCTCGATACA

Other Supporting Information Files

[Dataset S1 \(XLSX\)](#)