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

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Edited by Frank Van Breusegem, Vlaams Instituut voor Biotechnologie-Universiteit Gent, Ghent, Belgium, and accepted by the Editorial Board August 20, 2012 (received for review February 6, 2012)

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 ($^1O_2$) 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 $^1O_2$-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 $^1O_2$-responsive *AAA-ATPase* gene (*At3g28580*) and isolating second-site mutations that lead to a constitutive up-regulation of the reporter gene or abrogate its $^1O_2$-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 $^1O_2$-responsive transcripts in response to $^1O_2$. Topo VI binds to the promoter of the *AAA-ATPase* and other $^1O_2$-responsive genes, and hence could directly regulate their expression. Under photoinhibitory stress conditions, which enhance the production of $^1O_2$ and H_2O_2, Topo VI regulates $^1O_2$-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 ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (6, 7), whereas the analysis of singlet oxygen ($^1O_2$) and hydroxyl radical ($^{\bullet}OH$) 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 $^1O_2$ been shown to be involved in plastid-to-nucleus retrograde signaling. Immediately after the onset of $^1O_2$ generation in plastids of *Arabidopsis*, changes in nuclear gene expression reveal a rapid transfer of $^1O_2$-derived signals from the plastid to the nucleus (8–10). Several $^1O_2$-responsive genes are different from those activated by $O_2^{\bullet-}$ or H_2O_2, suggesting that $O_2^{\bullet-}/H_2O_2$- and $^1O_2$-dependent signaling occurs via distinct pathways (8, 10, 11). Other consequences of increased $^1O_2$ generation inside plastids include a drastic reduction in the growth rate of mature plants and the bleaching and death of seedlings (10). All these $^1O_2$-mediated responses are genetically regulated by the two plastid proteins, EXECUTER1 and EXEXUTER2, required for the translocation of $^1O_2$-derived signals from the plastid to the nucleus (12, 13).

$^1O_2$ 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 $^1O_2$-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 $^1O_2$-dependent up-regulation (14). The reporter gene consisted of the luciferase ORF and the promoter of the $^1O_2$-responsive *AAA-ATPase* nuclear gene of *Arabidopsis* (8, 10). Here we report on the identification and characterization of mutant *caa39*, whose response to $^1O_2$ 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 $^1O_2$-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 $^1O_2$-responsive *AAA-ATPase* promoter (14), was mutagenized and screened for second-site mutants that either constitutively up-regulate the $^1O_2$-responsive reporter gene or have lost the ability to respond

Author contributions: K.S., K.A., and C.L. designed research; K.S., F.M., P.P., C.V., A.B., C.A., and C.L. performed research; K.S., F.M., P.P., C.V., L.H., K.A., and C.L. analyzed data; and K.S., L.H., K.A., and C.L. wrote the paper. F.M., P.P. and C.V. contributed equally to this work.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. F.V.B. is a guest editor invited by the Editorial Board.

Data deposition: The microarray data have been deposited in ArrayExpress, www.ebi.ac.uk/arrayexpress, (ID no. E-TABM-1076).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1202041109/-/DCSupplemental.

to $^1O_2$. 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 $^1O_2$-signaling pathway and that also contain the *flu* mutation, generation of $^1O_2$ by a dark-to-light (D/L) shift should not further enhance the constitutive expression of $^1O_2$-responsive genes. Based on this criterion, only one of the six *caa* mutants, *caa39*, could be directly linked to $^1O_2$ signaling (14–16). Until 5 d old, *caa39* mutant seedlings grown under continuous light were phenotypically similar to wild-type seedlings (Fig. 1*A*). Afterward they developed chlorotic spots and spontaneous cell death, and their growth slowed relative to wild-type (Fig. 1 *A* 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. 1*C* 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. 1*D*). *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. 1*D*). 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. 1*E*). 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 A_2B_2 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 $^1O_2$-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 $^1O_2$-responsive gene expression while being part of the Topo VI complex.

AtTOP6A Regulates $^1O_2$-Triggered Stress Responses. To confirm that AtTOP6A represents a genuine $^1O_2$ signaling component, we further analyzed the *flu caa39* mutant under $^1O_2$-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 (Pchlide) in the dark and, hence, do not release $^1O_2$ 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. 1 *A* and *B*). Following a D/L shift, $^1O_2$-induced accumulation of *AAA-ATPase* and *BAP1* transcripts was strongly suppressed in *flu caa39* seedlings (Fig. 3*A*), even though they accumulated similar excess amounts of Pchlide as the parental *flu* line (14); a full induction was restored in complemented *flu caa39 35S:AtTOP6A* plants (Fig. 3*A*). The impaired $^1O_2$-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 $^1O_2$ production because: (*i*) 5-d-old *flu caa39* seedlings only moderately accumulated *AAA-ATPase* transcripts compared with 10-d-old seedlings (Fig. 1*B*), 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 $^1O_2$ in 5-d-old seedlings (14–16).

It is conceivable that Topo VI may also regulate the expression of other $^1O_2$-responsive genes and affect phenotypic changes, such as cell death, that are triggered by the release of $^1O_2$ in the *flu* mutant (10). Therefore, we tried to identify genes that were affected by the *caa39* mutation before and after the release of $^1O_2$. 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 $^1O_2$. 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. 3*B*). 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 e^{-06}] (Dataset S1), indicating that although no cell death could be detected in 5-d-old *flu caa39* seedlings under continuous light (Fig. 3*C*), 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 $^1O_2$. 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 $^1O_2$

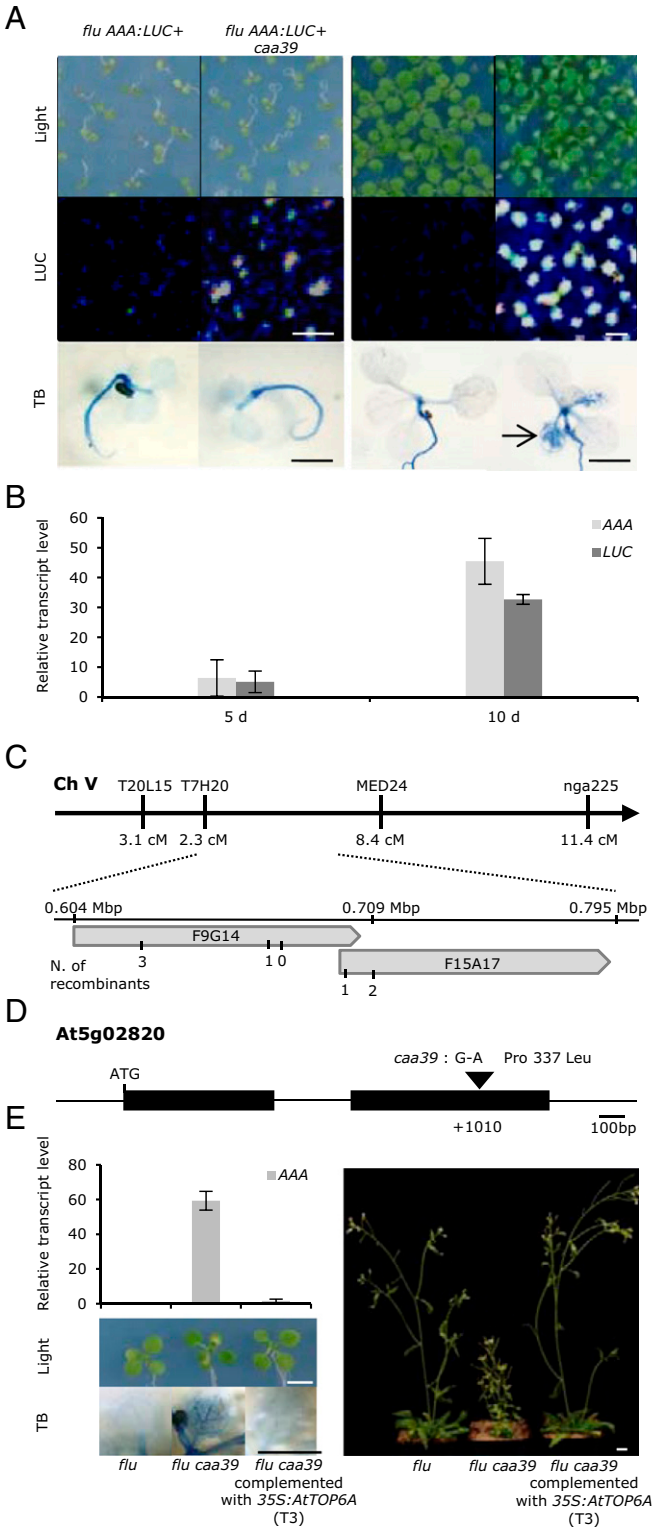

Fig. 1. Identification of the *CAA39* gene. (*A*) Constitutive luciferase activity and phenotype of the *flu AAA:LUC*$^+$ *caa39* and the *flu AAA:LUC*$^+$ parental line. Seedlings were grown in continuous light on Murashige and Skoog agar plates for 5 d and 10 d. Bioluminescence (LUC), corresponding 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 ± SE. (*C*) Map-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 *At5g28020* 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 *At5g28020* gene. (Scale bars, 0.5 cm.)

in a Topo VI-dependent manner) (Fig. 3*B* and Dataset S1). Conversely, genes in cluster 5 (Fig. 3*B* 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 e^{-04}). 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. 3*B* 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 subfractions of $^1O_2$-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 $^1O_2$ generated by a D/L shift, we found that the cell-death response was significantly reduced compared with the parental line (Fig. 3 *C* and *D*).

Contrasting Roles of AtTOP6A During Expression Changes of $^1O_2$- 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 photoinhibition and increases the production of $^1O_2$ and H_2O_2 (27). We analyzed the expression of the *AAA-ATPase* and other $^1O_2$-induced genes that are selectively activated by $^1O_2$ from the three main clusters [i.e., *At1g24145* 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-μmol photons $m^{-2}\cdot s^{-1}$ before transferring them to high light (2,000 $\mu mol\cdot m^{-2}\cdot s^{-1}$). In wild-type plants, transcripts of the $^1O_2$-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 $^1O_2$ and H_2O_2-responsive genes (Fig. 4). In *caa39*, induction of the $^1O_2$-responsive genes was reduced (Fig. 4), except *ERF5* that was also unaffected by the *caa39* mutation in the *flu* mutant (Fig. 3*B*, 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 $\mu mol\cdot m^{-2}\cdot s^{-1}$) (Fig. S4). These results suggest that AtTOP6A can act as a positive regulator of $^1O_2$-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 $^1O_2$-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

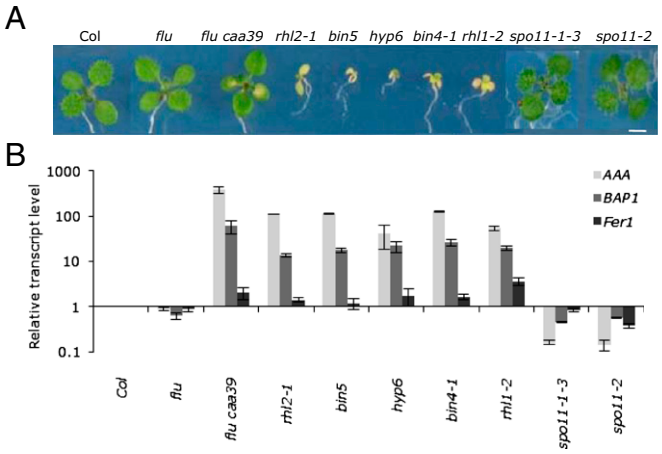

Fig. 2. Phenotype of mutants with impaired *Arabidopsis* Topo VI subunits. (*A*) Visible phenotypes of 10-d-old seedlings grown under continuous light. (*B*) Relative transcript levels of endogenous *AAA-ATPase* (*AAA*), *BAP1* and *FER1* marker genes were analyzed in cotyledons of 10-d-old seedlings 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 ± 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 $^1O_2$-responsive gene transcription.

Discussion

Topo VI, A Genuine Component of $^1O_2$ Retrograde Signaling. The *caa39* mutant was isolated in a genetic screen aimed at identifying factors involved in $^1O_2$-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 $^1O_2$ production (14). The characterization of these mutants suggested that $^1O_2$-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 $^1O_2$-signaling. *caa39* was the only *caa* mutant that showed an impaired $^1O_2$-induced accumulation of *AAA-ATPase* transcripts and other $^1O_2$-responsive genes, and hence AtTOP6A seems to form a genuine part of $^1O_2$ 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 $^1O_2$-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 $^1O_2$ is unlikely to leave the chloroplast in *Arabidopsis* (13), this observation supports that several $^1O_2$-derived signals might be

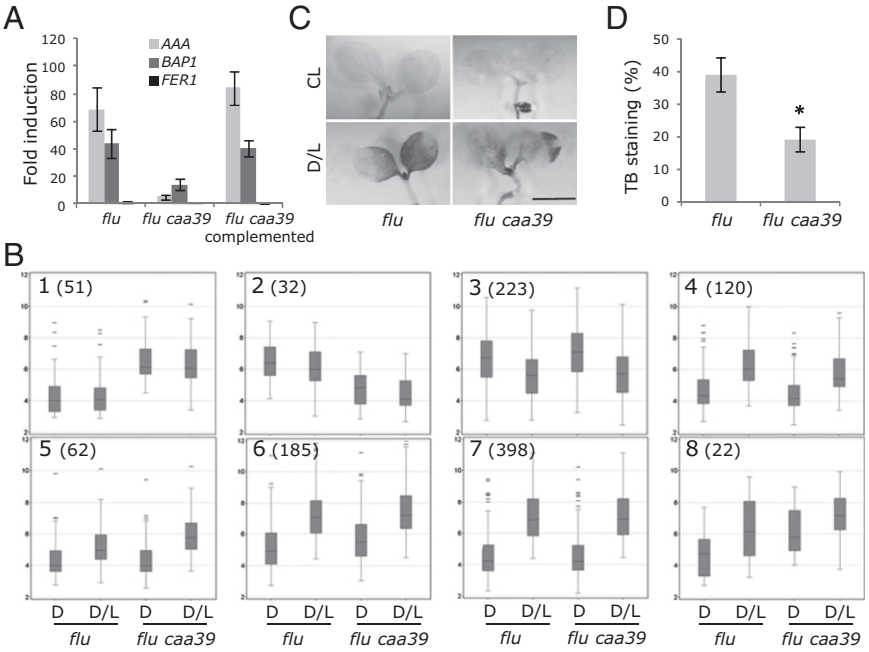

Fig. 3. Response of the *flu caa39* mutant to the release of singlet oxygen. (*A*) Fold-inductions of *AAA-ATPase*, *BAP1*, and *FER1* in *flu*, *flu caa39*, and *flu caa39* complemented with the wild-type copy of the *AtTOP6A* gene. The 5-d-old seedlings were subjected to 8 h dark and 30 min reillumination (D/L). Transcript levels were determined by quantitative RT-PCR. Results represent mean values of two biological replicates ± SE (*B*) Genome-wide identification of 1,093 genes differently regulated in *flu* or *flu caa39* prior or after the release of $^1O_2$. 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 ± SE). *t test $P < 0.01$.

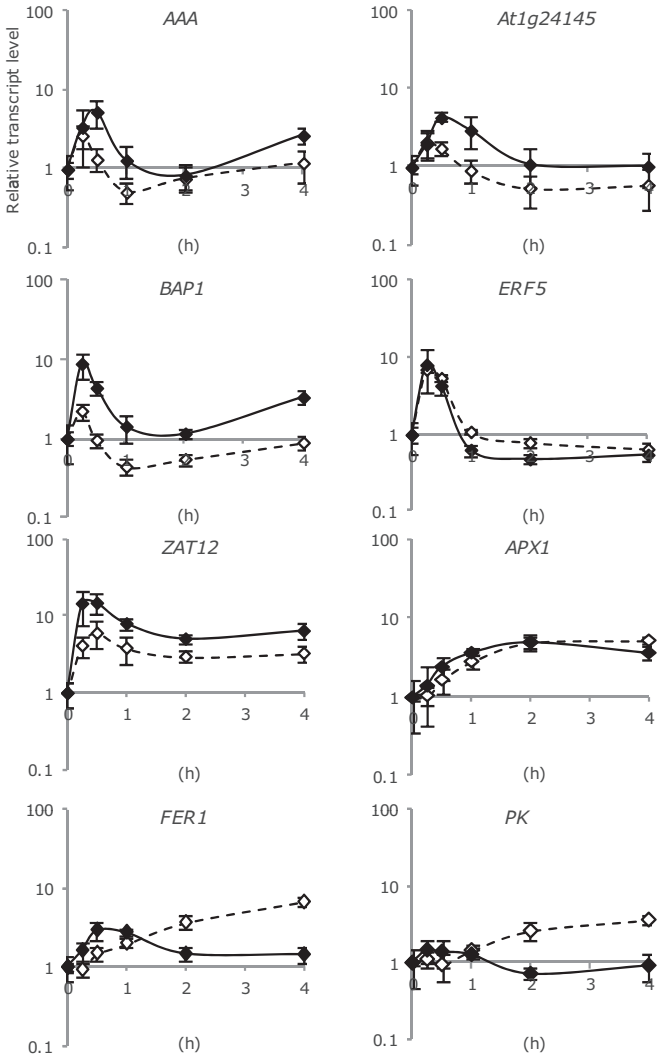

Fig. 4. Response of the wild-type Col and *caa39* mutant to high-light stress conditions. Relative transcript levels of the $^1O_2$-responsive *AAA-ATPase*, *At1g24145*, *BAP1*, *ERF5*, the H_2O_2-responsive *FER1*, *PK*, and *APX1* genes, and the general ROS-responsive marker gene *ZAT12* were analyzed 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 ± 50 μmol·m^{-2} s^{-1}) treatment. Before HL stress, seedlings were grown for 5 d in normal light (NL, 80 ± 5 μmol·m^{-2}·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 ± 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 $^1O_2$-responsive genes. A candidate $^1O_2$-derived signal could be β-cyclocitral, a carotenoid oxidation product that was recently found to accumulate under high light conditions that generate $^1O_2$ (30). However, because β-cyclocitral–mediated signaling does not appear to require the EXECUTER1 function, it seems to be different from the EXECUTER1-dependent $^1O_2$-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 $^1O_2$. 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 $^1O_2$-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., $^1O_2$-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 $^1O_2$-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 hTopoI 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 $^1O_2$-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 IIβ 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.

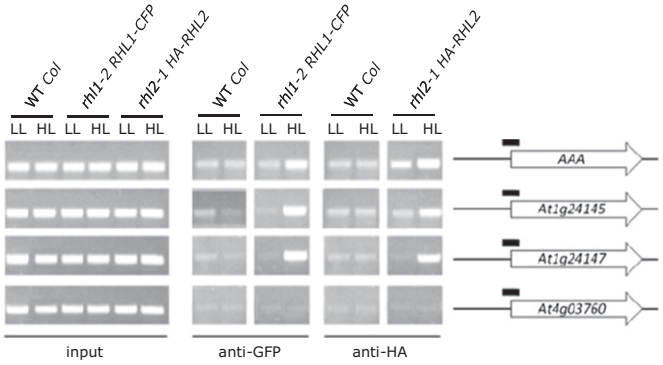

Fig. 5. Identification of Topo VI binding sites in vivo. ChIP assays were carried out using anti-GFP and anti-HA antibodies with wild-type (Col), *rhl1-2 RHL1-CFP*, and *rhl2-1 HA-RHL2* plants that were grown for 6 d in low light (LL; 12 μmol·m^{-2}·s^{-1}) and then transferred to moderate high light (HL; 1,050 μmol·m^{-2}·s^{-1}) for 30 min. PCR analysis was carried out to probe RHL1 and RHL2 binding to the promoter region of the $^1O_2$-responsive *AAA-ATPase*, *At1g24145*, and *At1g24147* genes and the control locus *At4g07360*. 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 $^1O_2$-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*.

Luciferase Imaging, Trypan Blue Staining, and Quantification of Cell Death. Luciferase imaging in plants was performed as previously described (14). Dead cells were identified by staining with lacto-phenol Trypan blue (41). Seedlings were photographed and the area of positive Trypan blue staining was quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

ChIP. ChIP was performed as previously described (42). Anti-GFP antibodies (11 814 460 001; Roche), anti-HA antibodies (11 867 423 001; Roche), antidimethyl-histone H3 Lys-9 antiserum (07-690; Upstate), and anti-IgG antibody (I5381; Sigma-Aldrich) were used for immunoprecipitation. Immunoprecipitated DNA was analyzed by PCR using *AAA-ATPase*, *At1g24145*, and *At1g24147* gene-specific primers and primers against the control locus *At4g03760* (Tables S1–S5).

Identification and complementation of the *caa39* mutation, RNA isolation, quantitative RT-PCR, and microarray analysis are described in *SI Materials and Methods*. See Fig. S7 for validation of reference genes.

ACKNOWLEDGMENTS. We thank André Imboden, Mena Nater, and Geoffrey Foucher for technical assistance, and Dr. Ben Field for critical reading of the manuscript. This study was supported by the Eidgenössiche Technische Hochschule Zurich; the Swiss National Science Foundation; the Functional Genomic Center Zürich; National Institutes of Health Grant R01-GM085036 (to K.A.); Swiss National Science Foundation Grant 31003A_13027 (to L.H.); and French National Research Agency Grant ANR 2010 JCJC 1205 01 (to C.L.).

1. Miller G, Shulaev V, Mittler R (2008) Reactive oxygen signaling and abiotic stress. *Physiol Plant* 133:481–489.
2. Apel K, Hirt H (2004) Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55:373–399.
3. Gechev TS, Van Breusegem F, Stone JM, Denev I, Laloi C (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *Bioessays* 28:1091–1101.
4. Laloi C, Apel K, Danon A (2004) Reactive oxygen signalling: The latest news. *Curr Opin Plant Biol* 7:323–328.
5. Møller IM, Jensen PE, Hansson A (2007) Oxidative modifications to cellular components in plants. *Annu Rev Plant Biol* 58:459–481.
6. Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant* 11:1187–1194.
7. Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79:583–593.
8. Laloi C, Przybyla D, Apel K (2006) A genetic approach towards elucidating the biological activity of different reactive oxygen species in *Arabidopsis thaliana*. *J Exp Bot* 57:1719–1724.
9. Meskauskiene R, et al. (2001) FLU: A negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 98:12826–12831.
10. op den Camp RG, et al. (2003) Rapid induction of distinct stress responses after the release of singlet oxygen in *Arabidopsis*. *Plant Cell* 15:2320–2332.
11. Laloi C, et al. (2007) Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 104:672–677.
12. Lee KP, Kim C, Landgraf F, Apel K (2007) EXECUTER1- and EXECUTER2-dependent transfer of stress-related signals from the plastid to the nucleus of *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 104:10270–10275.
13. Wagner D, et al. (2004) The genetic basis of singlet oxygen-induced stress responses of *Arabidopsis thaliana*. *Science* 306:1183–1185.
14. Baruah A, Simková K, Apel K, Laloi C (2009) *Arabidopsis* mutants reveal multiple singlet oxygen signaling pathways involved in stress response and development. *Plant Mol Biol* 70:547–563.
15. Baruah A, Simková K, Hincha DK, Apel K, Laloi C (2009) Modulation of O-mediated retrograde signaling by the PLEIOTROPIC RESPONSE LOCUS 1 (PRL1) protein, a central integrator of stress and energy signaling. *Plant J* 60:22–32.
16. Šimková K, et al. (2012) The chloroplast division mutant *caa33* of *Arabidopsis thaliana* reveals the crucial impact of chloroplast homeostasis on stress acclimation and retrograde plastid-to-nucleus signaling. *Plant J* 69:701–712.
17. Sugimoto-Shirasu K, Stacey NJ, Corsar J, Roberts K, McCann MC (2002) DNA topoisomerase VI is essential for endoreduplication in *Arabidopsis*. *Curr Biol* 12:1782–1786.
18. Malik SB, Ramesh MA, Hulstrand AM, Logsdon JM, Jr. (2007) Protist homologs of the meiotic *Spo11* gene and topoisomerase VI reveal an evolutionary history of gene duplication and lineage-specific loss. *Mol Biol Evol* 24:2827–2841.
19. Bergerat A, Gadelle D, Forterre P (1994) Purification of a DNA topoisomerase II from the hyperthermophilic archaeon *Sulfolobus shibatae*. A thermostable enzyme with both bacterial and eucaryal features. *J Biol Chem* 269:27663–27669.
20. Corbett KD, Benedetti P, Berger JM (2007) Holoenzyme assembly and ATP-mediated conformational dynamics of topoisomerase VI. *Nat Struct Mol Biol* 14:611–619.
21. Forterre P, Gribaldo S, Gadelle D, Serre MC (2007) Origin and evolution of DNA topoisomerases. *Biochimie* 89:427–446.
22. Schneider K, et al. (1998) The *ROOT HAIRLESS 1* gene encodes a nuclear protein required for root hair initiation in *Arabidopsis*. *Genes Dev* 12:2013–2021.
23. Sugimoto-Shirasu K, et al. (2005) RHL1 is an essential component of the plant DNA topoisomerase VI complex and is required for ploidy-dependent cell growth. *Proc Natl Acad Sci USA* 102:18736–18741.
24. Breuer C, et al. (2007) BIN4, a novel component of the plant DNA topoisomerase VI complex, is required for endoreduplication in *Arabidopsis*. *Plant Cell* 19:3655–3668.
25. Kirik V, Schrader A, Uhrig JF, Hulskamp M (2007) MIDGET unravels functions of the *Arabidopsis* topoisomerase VI complex in DNA endoreduplication, chromatin condensation, and transcriptional silencing. *Plant Cell* 19:3100–3110.
26. Stacey NJ, et al. (2006) Arabidopsis SPO11-2 functions with SPO11-1 in meiotic recombination. *Plant J* 48:206–216.
27. Fryer MJ, Oxborough K, Mullineaux PM, Baker NR (2002) Imaging of photo-oxidative stress responses in leaves. *J Exp Bot* 53:1249–1254.
28. Danon A, Miersch O, Felix G, Camp RG, Apel K (2005) Concurrent activation of cell death-regulating signaling pathways by singlet oxygen in *Arabidopsis thaliana*. *Plant J* 41:68–80.
29. Gadjev I, et al. (2006) Transcriptomic footprints disclose specificity of reactive oxygen species signaling in *Arabidopsis*. *Plant Physiol* 141:436–445.
30. Ramel F, et al. (2012) Carotenoid oxidation products are stress signals that mediate gene responses to singlet oxygen in plants. *Proc Natl Acad Sci USA* 109:5535–5540.
31. Kim C, et al. (2012) Chloroplasts of *Arabidopsis* are the source and a primary target of a plant-specific programmed cell death signaling pathway. *Plant Cell* 24:3026–3039.
32. Brou C, et al. (1993) Sequence-specific transactivators counteract topoisomerase II-mediated inhibition of *in vitro* transcription by RNA polymerases I and II. *Nucleic Acids Res* 21:4011–4018.
33. McNamara S, Wang H, Hanna N, Miller WH, Jr. (2008) Topoisomerase IIbeta negatively modulates retinoic acid receptor alpha function: A novel mechanism of retinoic acid resistance. *Mol Cell Biol* 28:2066–2077.
34. Merino A, Madden KR, Lane WS, Champoux JJ, Reinberg D (1993) DNA topoisomerase I is involved in both repression and activation of transcription. *Nature* 365:227–232.
35. Evans-Roberts KM, Breuer C, Wall MK, Sugimoto-Shirasu K, Maxwell A (2010) *Arabidopsis thaliana* GYRB3 does not encode a DNA gyrase subunit. *PLoS ONE* 5:e9899.
36. Szklarczyk D, et al. (2011) The STRING database in 2011: Functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res* 39(Database issue):D561–D568.
37. Ju BG, et al. (2006) A topoisomerase IIbeta-mediated dsDNA break required for regulated transcription. *Science* 312:1798–1802.
38. Jain M, Tyagi AK, Khurana JP (2006) Overexpression of putative topoisomerase 6 genes from rice confers stress tolerance in transgenic *Arabidopsis* plants. *FEBS J* 273:5245–5260.
39. Jain M, Tyagi AK, Khurana JP (2008) Constitutive expression of a meiotic recombination protein gene homolog, *OsTOP6A1*, from rice confers abiotic stress tolerance in transgenic *Arabidopsis plants*. *Plant Cell Rep* 27:767–778.
40. Hruz T, et al. (2008) Genevestigator V3: A reference expression database for the meta-analysis of transcriptomes. *Adv Bioinformatics*, 2008:Article ID 420747.
41. Keogh RC, Deverall BJ, Mcleod S (1980) Comparison of histological and physiological responses to *Phakopsora pachyrhizi* in resistant and susceptible soybean. *Trans Br Mycol Soc* 74:329–333.
42. Bowler C, et al. (2004) Chromatin techniques for plant cells. *Plant J* 39:776–789.