Extranuclear protection of chromosomal DNA from oxidative stress

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Eukaryotic organisms evolved under aerobic conditions subjecting nuclear DNA to damage provoked by reactive oxygen species (ROS). Although ROS are thought to be a major cause of DNA damage, little is known about the molecular mechanisms protecting nuclear DNA from oxidative stress. Here we show that protection of nuclear DNA in plants requires a coordinated function of ROS-scavenging pathways residing in the cytosol and peroxisomes, demonstrating that nuclear ROS scavengers such as peroxiredoxin and glutathione are insufficient to safeguard DNA integrity. Both catalase (CAT2) and cytosolic ascorbate peroxidase (APX1) play a key role in protecting the plant genome against photorespiratory-dependent H_2O_2-induced DNA damage. In *apx1/cat2* double-mutant plants, a DNA damage response is activated, suppressing growth via a WEE1 kinase-dependent cell-cycle checkpoint. This response is correlated with enhanced tolerance to oxidative stress, DNA stress-causing agents, and inhibited programmed cell death.

Arabidopsis | stress tolerance | hydrogen peroxide

Reactive oxygen species (ROS) are toxic molecules continuously produced in cells during aerobic metabolism. In plants ROS are produced mainly in peroxisomes during photorespiration, in chloroplasts during photosynthesis, and in mitochondria during respiration (1, 2). Unless detoxified by specialized enzymes and low molecular antioxidants, ROS can lead to protein, lipid, and DNA oxidation and to cell death (1, 2). Plants contain a large network of genes encoding different pathways involved in ROS scavenging and production, with a key role in managing the overall steady-state level of ROS in cells (2). Similar to genotoxic agents or ionizing radiation, ROS-derived DNA oxidation leads to altered bases and damaged sugar residues, resulting in DNA single- and double-strand breaks (3, 4). Strand breaks trigger a DNA damage response (DDR) by inducing the expression of molecular markers associated with DNA damage repair, such as poly(ADP ribose) polymerase (PARP), RAD51, and BREAST CANCER (BRCA) family members (5–8). Upon DNA stress, the ataxia telangiectasia-mutated (ATM) and the ataxia telangiectasia and Rad3-related (ATR) signaling kinases are activated and lead, via the WEE1 serine/threonine kinase, to a transient cell-cycle arrest that allows cells to repair DNA before proceeding into mitosis (9). Although oxidative DNA base damage has been shown to initiate a DDR in mammalian and yeast cells (10, 11), reports in plants on either the sources of oxidative stress that cause DNA damage or the subsequent induction of a DDR directly through ROS remain scarce (3, 12, 13). Until now, DNA damage and DDR in plants were studied mainly in response to exogenously applied DNA-damaging agents such as bleomycin and hydroxyurea or ionizing irradiation (9, 14, 15).

Protection against damage caused by ROS traditionally has been attributed to enzymes with ROS-detoxifying activities (16), and mutants lacking a particular ROS-scavenging enzyme were considered more sensitive to oxidative stress (17, 18). An unexpected finding, however, was that the lack of a cytosolic antioxidant enzyme (ascorbate peroxidase 1; APX1) was able to rescue, rather than worsen, the condition of a mutant lacking a peroxisomal ROS-scavenging enzyme (catalase 1; CAT1) in tobacco plants (19).

Here, we demonstrate that a DDR is induced in *Arabidopsis thaliana* double mutants lacking APX1 and CAT2 and that this response is correlated with an increased tolerance for agents causing oxidative stress and DNA stress. Our results indicate that a coordinated function of ROS-scavenging pathways in the cytosol and other cellular compartments is required for the protection of nuclear DNA, demonstrating that alternative nuclear ROS scavengers such as 1-cysteine peroxiredoxin, glutathione (GSH), and flavonoids (20–22) are insufficient to safeguard DNA integrity.

Results and Discussion

***Arabidopsis apx1/cat2* Double Mutant Is Protected Against Oxidative Stress.** We previously found that double-antisense tobacco plants deficient in both APX1 and CAT1 were less sensitive to oxidative stress than single-antisense plants lacking APX1 or CAT1 (19). To investigate whether this unexpected result is general to other plants and to identify some of the unknown mechanisms activated in these double mutants, we generated an *Arabidopsis thaliana* double mutant lacking APX1 and CAT2 (the equivalent of CAT1 in tobacco). In contrast to the single mutant *cat2*, the *apx1/cat2* double mutant was able to grow under high light (HL) conditions, did not accumulate ROS to detectable levels [visualized with diaminobenzidine (DAB) staining indicative of H_2O_2 accumulation], and had low levels of oxidized ribulose-1,5-bisphosphate carboxylase, indicating that the mechanism(s) activated in double mutants lacking cytosolic and peroxisomal H_2O_2-scavenging mechanisms are conserved (Fig.

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The authors declare no conflict of interest.

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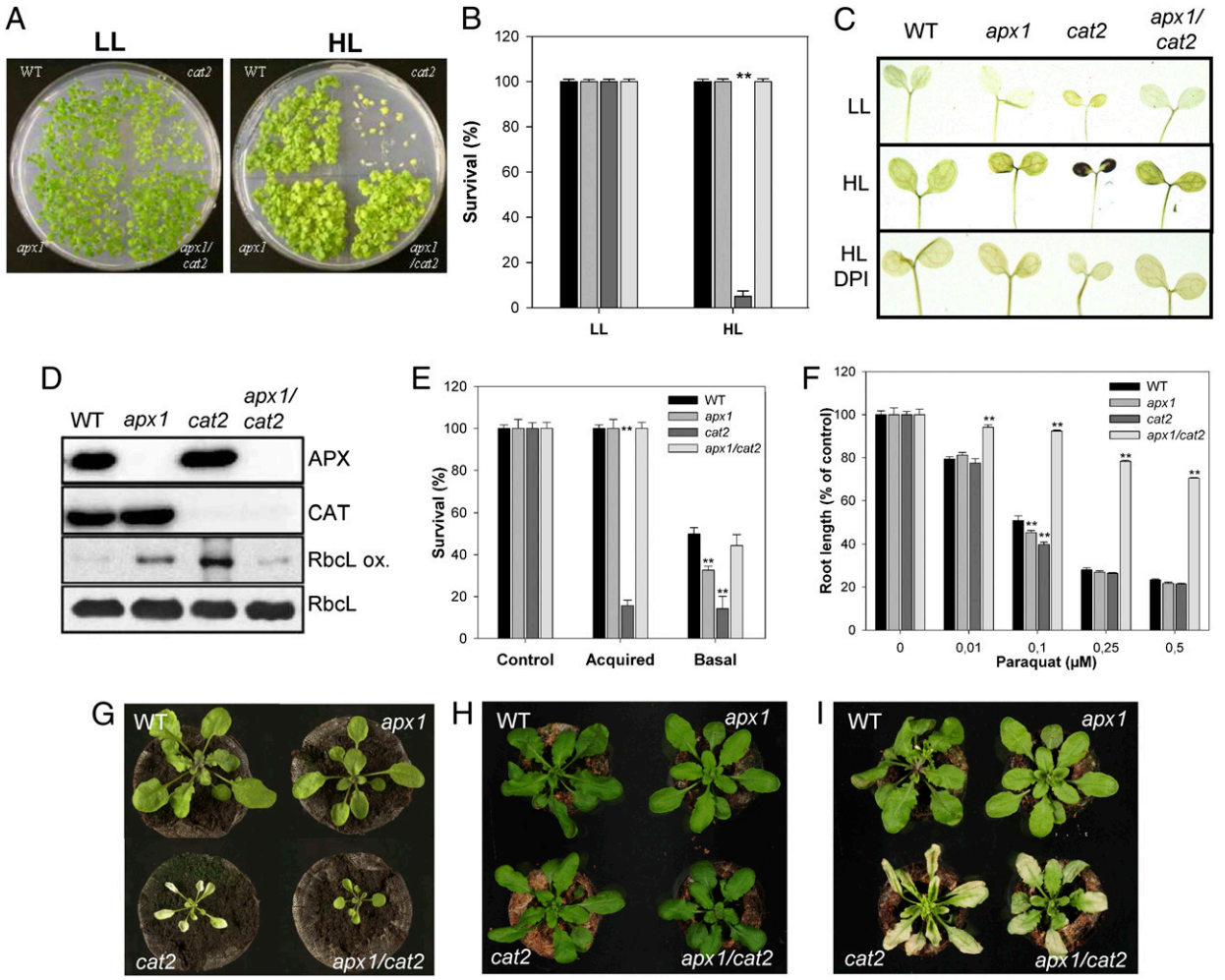

Fig. 1. Tolerance of *apx1/cat2* to oxidative stress. (*A* and *B*) Photographs (*A*) and survival rates (*B*) of WT (black bars), *apx1* (medium gray bars), *cat2* (dark gray bars), and *apx1/cat2* (light gray bars) seedlings grown under LL or HL conditions. (*C*) DAB staining (brown) indicating accumulation of H_2O_2 in seedlings grown at LL or subjected to HL stress in the absence or presence of 10 μM DPI (an inhibitor of NADPH oxidase). (*D*) Protein blot analysis of catalase (CAT) and ascorbate peroxidase (APX) in WT, *apx1*, *cat2*, and *apx1/cat2* plants and detection of ribulose-1,5-bisphosphate carboxylase (RbcL) protein oxidation in leaf extracts obtained from HL-treated plants (900 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 1 h). (*E*) Survival rates in response to heat stress, showing enhanced basal and acquired thermotolerance of *apx1/cat2* plants compared with *cat2* plants. (*F*) Root growth in the presence of increasing concentrations of paraquat. Root growth of WT, *apx1*, and *cat2* seedlings was severely reduced, but *apx1/cat2* plants displayed high levels of tolerance to oxidative stress. (*G*) Photograph of WT, *apx1*, *cat2*, and *apx1/cat2* plants grown under ambient air and exposed to HL stress (1,000 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 24 h). Lesions are apparent only on the leaves of *cat2* plants. (*H*) Photograph of 4-wk-old plants grown at high CO_2 (3,000 ppm). High CO_2 abolished growth retardation in *cat2* and *apx1/cat2* plants. (*I*) Photograph of plants grown in high CO_2 and transferred to ambient air and subjected to 24 h of HL stress. Lesions also appeared on leaves of *apx1/cat2* plants and were as prominent as in *cat2* plants. Error bars in *B*, *E*, and *F* show SEM ($n = 60$); $^{**}P < 0.01$ (Student's *t* test).

1*A*–*D*). Accumulation of ROS in *cat2* was suppressed by the ROS-generating NADPH oxidase inhibitor diphenyleneiodonium (DPI) (Fig. 1*C*), demonstrating that this process is a secondary event caused by the activation of a signaling pathway that involves ROS production via NADPH oxidase activity, rather than a primary cause of CAT2 deficiency. *cat2* was more sensitive to heat stress in both pretreated (acquired) and non-pretreated (basal) plants, and the lack of APX1 was able to compensate for this sensitivity (Fig. 1*E*). Moreover, the *apx1/cat2* mutant was more tolerant to oxidative stress imposed by the superoxide-generating herbicide paraquat than WT or single mutants (Fig. 1*F*). The signaling pathway activated in *apx1/cat2* therefore was functional against oxidative stress generated by at least three different treatments (HL, heat, and paraquat application).

When subjected to HL under ambient conditions, *cat2* and *apx1/cat2* plants were smaller than WT and *apx1* plants, but the double mutants did not develop lesions (Fig. 1*G*). To identify the subcellular source of ROS that triggers the mechanisms responsible for HL tolerance, plants were grown at high CO_2 concentrations, a treatment that abolishes ROS production in peroxisomes (23). Constant growth at high CO_2 suppressed growth retardation in *cat2* and *apx1/cat2* plants and lesion formation in *cat2* plants (Fig. 1*H*). In contrast to *apx1/cat2* plants grown under ambient conditions (Fig. 1*G*), *apx1/cat2* plants grown under high CO_2 and transferred to ambient air developed lesions (Fig. 1*I*). This result identified peroxisomal H_2O_2 as the ROS primarily responsible for the activation of defense and/or acclimation mechanisms in *apx1/cat2* plants and showed that growth in ambient air constitutively triggers the HL acclimation pathway in *apx1/cat2* mutants. Measurements of glutathione redox state concur with the results shown in Figs. 1 and 2 demonstrating that *cat2* plants grown in ambient air have an oxidized cellular redox state and that growth of these plants in high CO_2 prevents this oxidation. In contrast, *apx1/cat2* plants grown under ambient air or high CO_2 have a reduced cellular redox state (Table S1).

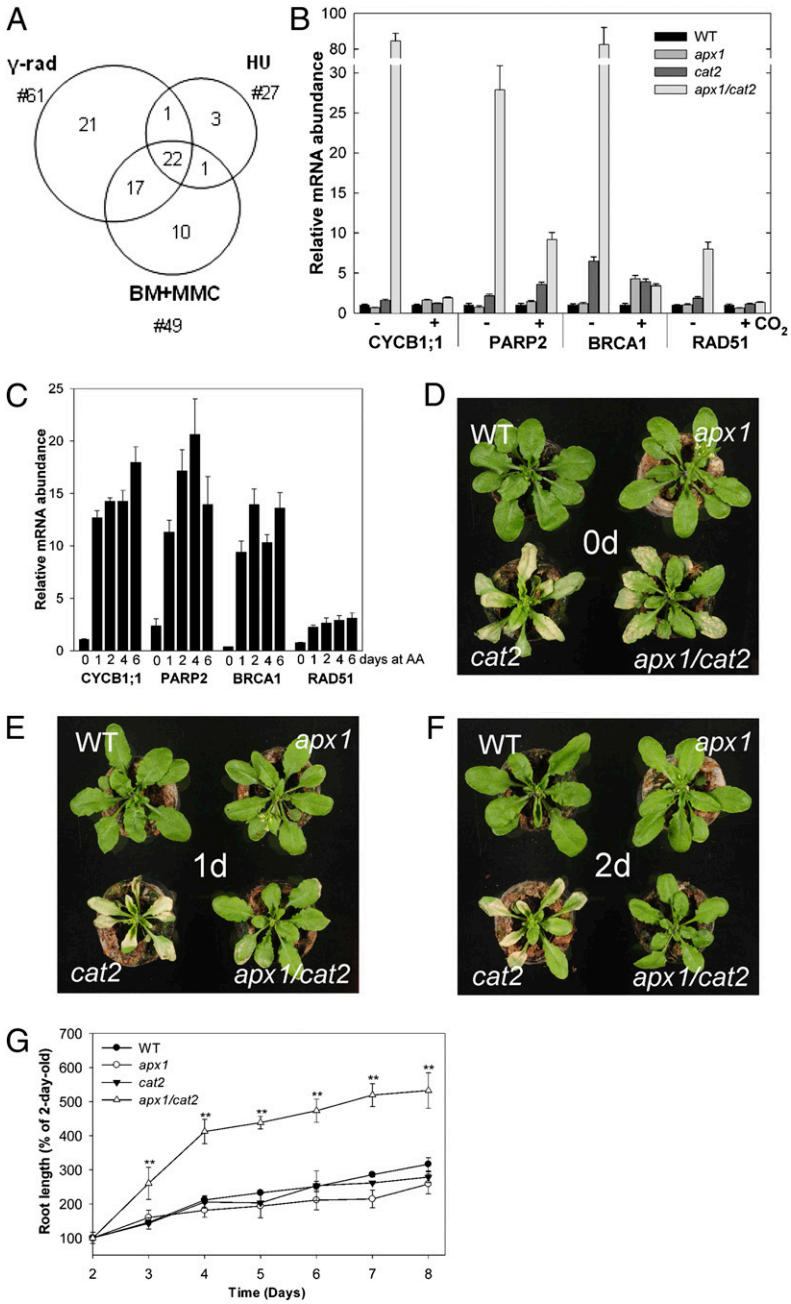

Fig. 2. Functionality of the H_2O_2-dependent DDR in *apx1/cat2* plants. (*A*) Venn diagram showing the overlap between constitutive *apx1/cat2*-specific transcripts and transcripts accumulating in cells in response to three different DNA stress-generating conditions: γ irradiation (γ-rad), hydroxyurea (HU), and a combination of bleomycin and mitomycin C (BM+MMC). In total, 75 *apx1/cat2*-specific transcripts were positively regulated in at least one DNA stress experiment, and 22 transcripts accumulated in all DNA stress experiments. (*B* and *C*) Accumulation of DNA stress marker transcripts (*CYCB1;1*, *PARP2*, *BRCA1*, and *RAD51*) in WT, *apx1*, *cat2*, and *apx1/cat2* plants grown in ambient air (–CO_2) or high CO_2 (+CO_2; 3,000 ppm) (*B*) and in *apx1/cat2* plants released from a high-CO_2 environment to ambient air (AA) at LL (*C*). (*D*–*F*) Photographs showing a time course in which different groups of WT, *apx1*, *cat2*, and *apx1/cat2* plants grown under high CO_2 were transferred to ambient air for 0 (*D*), 1 (*E*), or 2 d (*F*) at LL and subsequently treated with HL. (*G*) Root growth kinetics of WT, *apx1*, *cat2*, and *apx1/cat2* seedlings grown in the presence of aphidicolin (12 μg/mL). Error bars show SEM (n = 60). $^{**}P < 0.01$ (Student's t test).

DNA Damage Response Is Constitutively Induced in *apx1/cat2* Plants and Contributes to the Stress-Resistance Phenotype. A genome-wide transcriptome analysis of WT, *apx1, cat2,* and *apx1/cat2* plants grown under ambient air and exposed to HL for 0 or 1 h identified 381 transcripts that specifically and constitutively accumulated in *apx1/cat2* plants (Table S2 and Fig. S1). In contrast, no *apx1/cat2*-specific HL-induced transcripts were identified (Fig. S1). Interestingly, none of the 381 constitutively accumulating transcripts found in *apx1/cat2* plants corresponded to known enzymes with superoxide- or H_2O_2-scavenging activities (2) (Table S2). However, a significant enrichment for genes that previously had been reported to be induced by different genotoxic stresses, such as γ irradiation and hydroxyurea, including *BRCA1*, *PARP2*, B-type cyclin (*CYCB1;1*), and *RAD51*, which are typical hallmarks of the DDR (5–9, 14, 15), was found among the constitutively expressed transcripts in *apx1/cat2* mutants (see Fig. 2, *A* and *B* for mature plants and Fig. S2 for 8-d-old seedlings). When plants were grown under high CO_2 levels, which

suppressed ROS accumulation because of CAT2 deficiency, the accumulation of DDR transcripts in *apx1/cat2* plants was suppressed (Fig. 2*B*). In time-course experiments in which *apx1/cat2* plants were released from a high CO_2 environment to ambient air, DNA stress-responsive transcripts accumulated within 1 d, corresponding with the induction of the HL resistance phenotype in *apx1/cat2* plants (Fig. 2 *C–F*).

Functional DDR Is Activated in the Absence of Detectable DNA Lesions. To determine whether the DDR induced in *apx1/cat2* plants correlated with increased amounts of DNA damage, we examined both the levels of phosphorylated histone H2AX (γ-H2AX), a reliable indicator of DNA double-strand breaks (24, 25) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo), a hallmark of oxidative DNA base damage (26). No increase in γ-H2AX was observed in any of the lines (Fig. S3*A*), and compared with *cat2* plants grown in ambient air, *apx1/cat2* plants did not accumulate 8-oxo-dGuo (Fig. S3*B*), suggesting that activation of the DDR pathway might not require DNA damage (10, 27) or, alternatively, that DNA lesions are repaired efficiently, leading to levels of basal DNA damage similar to those in WT and *apx1* plants. The lack of DDR activation in *cat2* grown under ambient conditions suggests that DDR activation is highly regulated and requires more than one type or source of ROS signal(s). Furthermore, treatment with the DNA stress-causing agent aphidicolin revealed that *apx1/cat2* plants were more tolerant than WT, *apx1*, or *cat2* plants [Fig. 2*G*; see also Fig. S4 for tolerance to mitomycin C (MMC)], indicating that the constitutively activated DDR in *apx1/cat2* plants is functional.

WEE1-Dependent Checkpoint Suppresses Growth in *apx1/cat2* Plants. An inherent part of the DDR is the activation of checkpoints to arrest cell-cycle progression and to allow time for repair, thus preserving genome integrity (9, 28, 29). Conserved key regulators for these checkpoint pathways are the ATM, ATR, and WEE1 kinases (9, 28). Distribution profiles of DNA ploidy levels assessed in 9-d-old plants revealed a small but significant increase in 4C/2C cell ratios in *apx1/cat2* plants, indicating an impairment of cell-cycle progression at the G2-to-M transition (Fig. 3*A*). In accordance, *apx1/cat2* plants displayed a decreased average cell number and area per leaf (Fig. 3*B*). At the G2 checkpoint, WEE1 kinase is a critical downstream target of the ATM/ATR signaling cascade (9, 28). In cells suffering DNA damage, WEE1 halts cell-cycle progression upon cessation of DNA replication, thereby coupling mitosis to DNA repair (9).

To evaluate the role of WEE1 in *apx1/cat2* double mutants, we generated *apx1/cat2/wee1* plants. During the production of these triple mutants, we observed that the RNAi effect that down-regulated the *CAT2* transcript and subsequent activity levels was lost recurrently in individual progeny plants. These findings indicate that, in individual *apx1/cat2/wee1* mutants, unknown mechanisms are activated that interfere with RNAi-dependent gene silencing. Nevertheless, several individual catalase-deficient triple mutants could be identified through PCR-based genotyping and CAT activity assays. These individual triple mutants all showed restored growth capacity compared with *apx1/cat2* double mutants and also abolished transcriptional induction of the DDR (Fig. 3 *C* and *D*). Together with the DNA ploidy assays (Fig. 3*A*), these results revealed that WEE1-dependent checkpoints are activated as part of the H_2O_2-dependent DDR of *apx1/cat2* plants. The involvement of the DDR in cellular protection from oxidative stress also was evident from the enhanced sensitivity to HL and oxidative stress of the DNA stress checkpoint mutants *atr-2*, E2F target gene 1 (*etg1-1*), and *wee1-1* (Fig. 3 *E* and *F*) and from the identification of these DDR transcripts in plants treated with the herbicide norflurazon or infected with different pathogens (GENEVESTIGATOR; ref. 30); these results

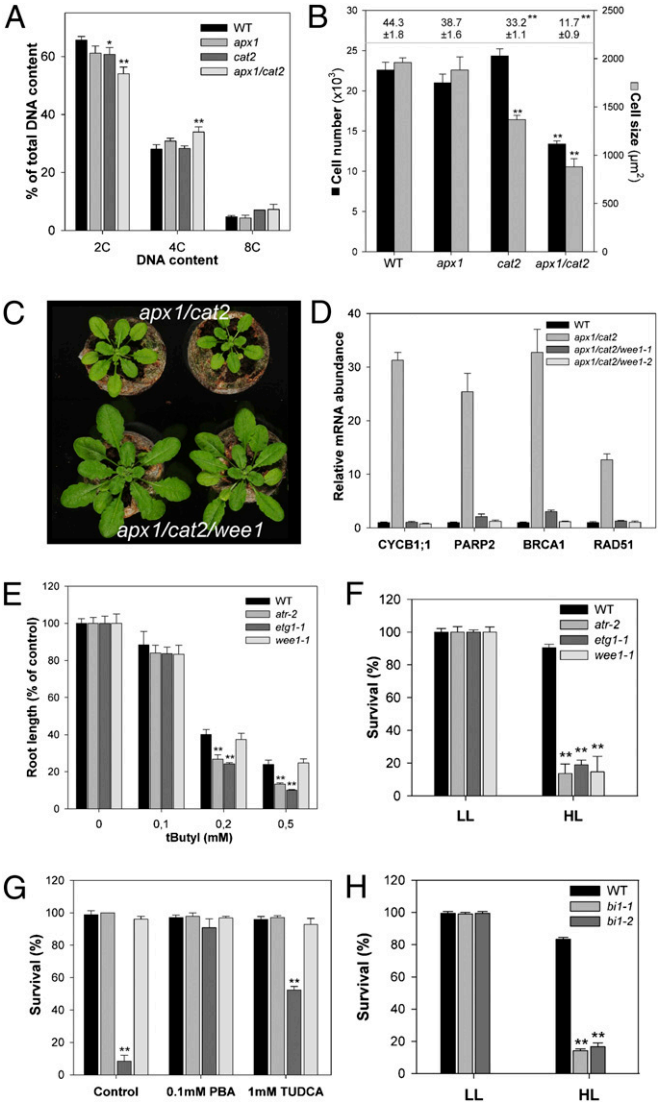

Fig. 3. Cell-cycle checkpoints and the ER–PCD pathway in *apx1/cat2* mutants and abiotic stress. (*A*) DNA ploidy-level distribution in the first leaves of 9-d-old seedlings. (*B*) Cell size and number in first leaves of 21-d-old seedlings. Average leaf blade area (±SEM) is shown at the top of the frame. (*C*) Photographs of *apx1/cat2* and *apx1/cat2/wee1* plants grown under ambient air conditions. (*D*) Accumulation of DNA stress marker transcripts (*CYCB1;1*, *BRCA1*, *PARP2*, and *RAD51*) in WT, *apx1/cat2*, and *apx1/cat2/wee1* plants. (*E*) Root growth of WT and DNA stress checkpoint mutants (*wee1-1*, *etg1-1*, and *atr-2*) in the presence of increasing concentrations of tertiary butyl hydroperoxide (tButyl). (*F*) Survival of WT, *wee1-1*, *etg1-1*, and *atr-2* plants grown under LL or HL conditions. (*G*) Survival rates of WT (black bar), *apx1* (medium gray bar), *cat2* (dark gray bar), and *apx1/cat2* (light gray bar) plants following HL stress in the absence (control) or presence of ER–PCD pathway–blocking agents PBA (0.1 mM) and TUDCA (1 mM). Application of PBA and TUDCA rescued *cat2* plants. (*H*) Survival rates of WT and *bi1* seedlings grown under LL or HL conditions. Error bars in *A*, *B*, and *E–H* show SEM (n = 3–5). $*P < 0.05$; $**P < 0.01$ (Student's t test).

demonstrate the broad biological role of the DDR in protecting plants from different environmental stresses.

The activation of the DDR in *apx1/cat2* plants also was coordinated with the accumulation of transcripts encoding Bcl2-associated X protein (Bax) inhibitor 1 (BI-1), a plant anti-programmed cell death (PCD) protein that suppresses the endoplasmatic reticulum (ER)–PCD pathway (31, 32). Application of agents that block the ER–PCD pathway rescued *cat2* under HL

conditions, and mutants deficient in BI-1 were highly sensitive to HL (Fig. 3 *G* and *H*), indicating that BI-1 is an important component of the pathways activated in *apx1/cat2* double mutants.

Conclusion

ROS play a multitude of biological roles in almost all organisms. ROS toxicity and its involvement in different human pathologies underline the need to identify new pathways and proteins that can mitigate the adverse effects of ROS accumulation (16, 33–37). It is surprising that, despite the widely assumed involvement of ROS in DNA damage in plants, no transcriptional DDR has been observed in various genome-wide transcript profiling studies that directly monitored ROS- or abiotic stress-mediated responses in *Arabidopsis* (38, 39). In sharp contrast to manifest oxidative lipid and protein modifications that occur upon ROS accumulation in cells (40, 41), the lack of a detectable DDR might reflect the existence of a highly efficient mechanism that preserves the nuclear plant genome against oxidative stress. Our results demonstrate that ROS produced in specific cellular compartments might reach the nuclei and trigger a DDR that is accompanied by activation of a cell-cycle checkpoint and that is essential to safeguard cells from oxidative stress (Fig. 4). This protective response requires a coordinated balance between different ROS-removal mechanisms residing outside the nuclei, i.e., in the cytosol and peroxisomes. Extranuclear protection of chromosomal DNA, BI-1, and the DDR therefore are important for the survival and growth of eukaryotic organisms under aerobic conditions.

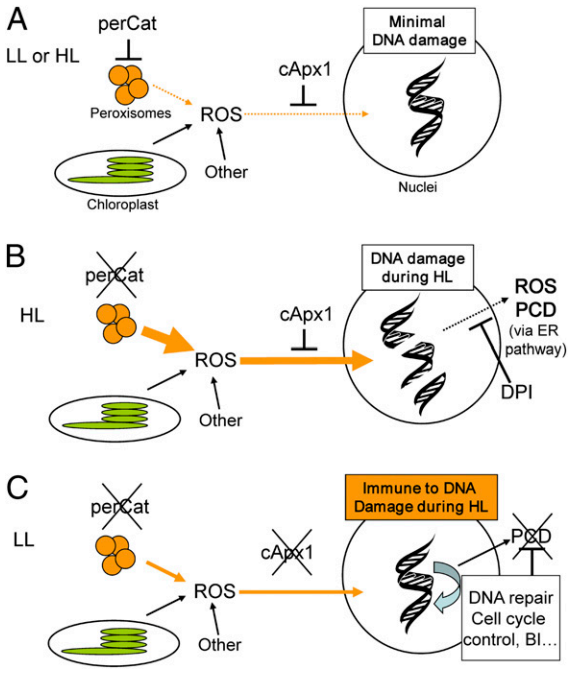

Fig. 4. Model for extranuclear protection of chromosomal DNA from oxidative stress. (*A*) ROS are maintained in cells by a network of scavenging enzymes such as cytosolic ascorbate peroxidase 1 (cApx1) and peroxisomal catalases (perCat) that protect cells against oxidative DNA damage. (*B*) Under HL conditions, a lack of perCat leads to the accumulation of ROS that overcomes the scavenging ability of cApx1 and triggers PCD that probably is mediated by the ER pathway. (*C*) The absence of both cApx1 and perCat in plants grown under LL triggers a network of DNA repair, cell-cycle control, and the ER–PCD pathway–suppressing BI-1 that renders cells highly tolerant of HL and oxidative stress conditions.

Materials and Methods

Plant Material and Growth Conditions. Knockdown *cat2* plants of *Arabidopsis thaliana* (L.) Heynh. (CAT2HP2) (18) were crossed with knockout *apx1* plants (17) and were self-fertilized. Double mutants were screened by genomic PCR analysis, protein gel blots, and CAT activity assays as described (17, 18). Homozygous plants were obtained for the four different configurations and were designated WT, *apx1*, *cat2*, and *apx1/cat2*. The *atr-2*, *wee1-1*, E2F target gene 1 (*etg1-1*), Bax inhibitor 1 (*bi1-1*), and *bi1-2* mutants have been described previously (9, 32, 42, 43). Plants were grown on Jiffy-7 soil pellets (Jiffy Products) in a controlled growth chamber (Weiss Umwelttechnik) at 21–22 °C, 16 h/8 h low-light (LL, 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) regime and 70% relative humidity or in vitro on half-strength Murashige and Skoog (1/2 M&S)-containing medium at 22 °C and 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in continuous light.

Stress Treatments and Assays. For HL treatment in soil, ≈3.5-wk-old plants (developmental stage 1.09–1.10) (44) were exposed to a continuous irradiation of ≈1,000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a growth cabinet (Sanyo). Heat, Paraquat, and tertiary butyl hydroperoxide stress treatments of seedlings were performed in growth chambers (E-30HB and AR-66; Percival Scientific) as described (45–47). For light-stress treatment, 7- to 9-d-old seedlings grown on 1/2 M&S agar plates under LL condition (25 $\mu mol \cdot m^{-2} \cdot s^{-1}$) were subjected to HL intensity (900 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 24–48 h and scored for survival. To determine effects of ER stress chaperons on the response of plants to light stress, seedlings grown as described above were sprayed with 0.1 mM sodium 4-phenylbutyrate (PBA), 1 mM tauroursodeoxycholic acid (TUDCA), or sterile distilled water (as a control) and were incubated at 22 °C for 3 h under LL condition (25 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Seedlings then were subjected to HL intensity (900 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 24–48 h and scored for survival. To study the effects of DNA stress-causing agents on the growth of seedlings, aphidicolin and MMC treatments were carried out as described (42, 48) with minor modifications. Briefly, seeds were placed on 1/2 M&S agar plates containing 12 μg/mL aphidicolin or 10 μg/mL MMC and were germinated as described (46). Root length was measured every 2 d following germination.

Microarray Analysis. Duplicate batches of middle-aged leaves of 25 WT, *apx1*, *cat2*, and *apx1/cat2* plants (developmental growth stage 1.09–1.10; ref. 44) subjected to 0 and 1 h of HL irradiation were harvested for total RNA preparation. Details on RNA preparation, microarray hybridization, data processing, and statistical analysis are provided in *SI Materials and Methods*. Full access to the microarray data is available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=fpahtwcuausemxw&acc=GSE19857).

Quantitative RT-PCR. Technical details and primers used for the quantitative RT-PCR analysis are provided in *SI Materials and Methods*.

Microarray Meta-Analysis. Transcripts specifically altered in *apx1/cat2* plants were compared with different DNA stress experiments. For γ irradiation, data of two different published datasets were combined. In the first experiment, genes showing a significant induction in WT plants (posterior probabilities of differential expression values >95% and *Q* value <0.05) after 1.5 h of γ irradiation with 100 Gy (14) were selected. In the second experiment, genes with a statistically significant induction (Bonferroni *P* value ≤0.05) in WT and an invariant expression or down-regulation in *atm* plants 5 h postirradiation (100 Gy) (15) were selected. For bleomycin and mitomycin stress, the GeneChip data were retrieved from the international AtGenExpress repository (Abiotic stress time series, Genotoxic stress; Kudla's laboratory). Data were processed as described (49), and a dataset of genes showing at least twofold induction after 12 h of genotoxic stress was created. For hydroxyurea stress, genes showing a significant induction (*t* test *P* value <0.05) in 7-d-old WT roots after 24 h of 2 mM hydroxyurea treatment were selected for comparative analysis.

Microscopy and Flow Cytometric Analyses. The distribution of nuclear DNA content was determined as described (43). At least two biological and three technical replicates were used for each sample analyzed. Size and number of abaxial pavement cells in leaves were determined as described (9).

Protein Oxidation Assay. Protein oxidation in extracts from plants subjected to HL stress for 1 h was determined by measuring the degree of protein carbonylation as described (45).

ROS Staining. Five-day-old seedlings grown in liquid 1/2 M&S medium under constant agitation were exposed to HL intensity (900 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 1 h,

immersed in 1 mg/mL DAB, 1/2 M&S solution, and fixed and imaged as described (45).

DNA Damage Detection. Details of 8-oxo-dGuo detection in DNA are provided in *SI Materials and Methods*. Histone extraction and immunoblotting for the detection of γ-H2AX were performed as described (25).

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