1 ml of NP-40 buffer [150 mM NaCl, 1.0% NP-40, 50 mM tris (pH 8.0)] containing protease inhibitors, and were precleared with protein A–Sepharose beads that had been incubated with normal rabbit serum. Cellular proteins were immunoprecipitated in the dark with 5 μg of an anti–caspase-3 IgG2a monoclonal antibody (Transduction Labs), 20 μl of an anti–caspase-3 rabbit polyclonal antiserum (Pharmingen), 5 μg of a control IgG2a antibody (Sigma), or control normal rabbit serum. The antibody/antigen complexes were isolated with protein A–Sepharose beads (Pharmacia) and then washed five times in high-salt buffer [500 mM NaCl, 1% NP-40, 50 mM tris (pH 8), 100 μM EDTA and protease inhibitors]. Antigen/antibody complexes were removed from the protein A–Sepharose beads by three 10-min incubations in 70 μl of 5 M $MgCl_2$ or 100 mM glycine (pH 3) at 4°C before NO measurements. To minimize the possibility of S-nitrosylation subsequent to immunoprecipitation, we raised the buffer pH to 5.5 in selected experiments and cleansed the solutions of contaminant nitrite by heating in sealed vessels for 2 hours at 95°C, at pH of 2.5 to 3.0. Any residual nitrite in buffers did not correlate with amounts of SNO in samples ($n = 55$, $R^2 = 0.03$). In addition, we adapted a methodology designed to exclude the possibility of artifactual S-nitrosylation by blocking free thiols in caspase-3 immunoprecipitates with 1 mM *N*-ethylmaleimide (NEM), included in wash buffers preceeding elution. Prior exposure to NEM completely blocked NO donor (pH 8)– or nitrite (0.5 N HCl)–mediated S-nitrosylation of recombinant caspase-3 or procaspase-3. Although NEM modestly reduced the NO signal derived from immunoprecipitated caspase-3 (~25%, $n = 7$), it also reduced by a similar amount the NO signal from highly pure SNO–caspase-3 [0.5 M NaCl, 1% NP-40, 50 mM tris (pH 8), 0.1 mM EDTA] that had been synthesized in vitro. Thus, this approach demonstrated that caspase-3 was S-nitrosylated intracellularly. For protein immunoblot analysis, whole-cell lysates or immunoprecipitated proteins were separated on 7% (NOS) or 12% (caspase-3) polyacrylamide gels, transferred to nitrocellulose, and incubated with 250 ng/ml of anti–caspase-3 monoclonal antibody, anti-nNOS monoclonal antibody, or anti-iNOS polyclonal antibody (Transduction Labs), or a 1:1,000 dilution of anti–caspase-3 rabbit polyclonal antibody (Pharmingen), followed by a 1:1000 dilution of secondary horseradish peroxidase antibody (Amersham), and then were developed by ECL (Amersham).

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. Photolysis-chemiluminesecence (assay for SNO) was performed as described [J. S. Stamler and M. Feelisch, in *Methods in Nitric Oxide Research*, J. S. Stamler and M. Feelisch, Eds. (Wiley, Chichester, UK, 1996), pp. 521–539]. Samples (200 μl) were injected directly into the photolysis unit. The chemical reduction–chemiluminescence assay for nitrite was performed according to the manufacturer (Sievers) and for SNO as described (*16*) with the following modifications: Immunoprecipitated proteins and cell extracts were assayed both with and without excess $HgCl_2$ in a paired radical-purged system, otherwise according to manufacturer's instructions (NO analyzer Sievers 280); standard curves were derived for both nitrite and S-nitrosoglutathione in the presence and absence of $HgCl_2$; data were normalized to baseline and integrated using Mathcad 7 Professional (MathSoft). SNO was calculated by subtracting the nitrite in samples without $HgCl_2$ from the nitrite in samples with $HgCl_2$ and by converting the nitrite difference to SNO from the standard curves of S-nitrosoglutathione (GSNO) ± $HgCl_2$. The photolysis and chemical reduction assays are linear to 1 pmol for SNO and nitrite, respectively, and can differentiate 1 pmol from zero (deionized water). S-Nitrosylated recombinant caspase-3 standards were detected by both methods; the standard curves for SNO–caspase-3 and GSNO were superimposeable in the photolysis assay. The chemical reduction method has not been extensively validated for SNO and thus was only used qualitatively to verify results from photolysis.

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11. We grew 9×10^7 MCF-7 cells to 75% confluence in T150 flasks containing Dulbecco's modified Eagle's medium supplemented with 10% FBS (Gemini), 2 mM glutamine (Cellgro), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL). The cells were then either transiently transfected with 120 μg of plasmids—engineered to express either wild-type or mutant procaspase-3 in which cysteine-163 was mutated to alanine (*9*), using lipofectAMINE per the manufacturer's protocol (Life Technologies)—or further treated with G418 to select for stable clones. The coding sequence of each expression construct was sequenced in its entirety before use. The cells were lysed in NP-40 buffer (48 hours following transient transfection), and caspase-3 immunoprecipitations were done as above. The level of wild-type and mutant caspase-3 in the lysates and immunoprecipitates was determined by protein immunoblot or silver stain analysis.

12. Fas was cross-linked on the surface of cells with 50 ng/ml of anti-Fas IgM clone CH-11 (Upstate Biotech) for 5 min to 2 hours at 37°C. The cells were then washed at 4°C with phosphate-buffered saline (PBS), and immunoprecipitations were done at selected intervals (*6*).

13. Cells were grown for 2, 24, or 48 hours in the presence or absence of the NO synthase inhibitor L-NMA (5 mM versus 1 mM L-arginine in the medium) (Calbiochem). The cells were then washed, and immunoprecipitations were done (*6*).

14. We grew 1×10^7 10C9 or Jurkat cells for 24 to 48 hours in the presence or absence of L-NMA as described above. Fas agonist antibody (100 ng/ml, clone CH-11, Upstate Biotech) and S-nitrosopenicillamine (500 μM) were then added to the appropriate cultures for 50 to 75 min, after which the cells were washed with ice-cold PBS and resuspended in 140 μl of buffer A [100 mM Hepes (pH 7.4), 140 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 10 μg/ml leupeptin]. The cells were then lysed with three cycles of freezing and thawing, and the crude cytosol was obtained by centrifugation at 12,000*g* for 20 min at 4°C. We mixed 50 to 200 μg of cytosolic protein with 400 μM Ac-DEVD-pNA (Quality Biochemicals) in 150 μl of buffer B [100 mM Hepes (pH 7.4), 20% glycerol, and protease inhibitors] and incubated it at 37°C. The caspase-3–like activity was calculated by measuring the increased absorbence at 405 nm every 10 min. The reaction mixture without cell lysate or substrate was used as a control.

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Systemic Signaling and Acclimation in Response to Excess Excitation Energy in *Arabidopsis*

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Land plants are sessile and have developed sophisticated mechanisms that allow for both immediate and acclimatory responses to changing environments. Partial exposure of low light–adapted *Arabidopsis* plants to excess light results in a systemic acclimation to excess excitation energy and consequent photooxidative stress in unexposed leaves. Thus, plants possess a mechanism to communicate excess excitation energy systemically, allowing them to mount a defense against further episodes of such stress. Systemic redox changes in the proximity of photosystem II, hydrogen peroxide, and the induction of antioxidant defenses are key determinants of this mechanism of systemic acquired acclimation.

Large increases in light intensity for a short period can be beneficial for photosynthetic yields in low light (LL)–adapted plants (*1*). However, if these conditions persist, an imbalance can be created such that the energy absorbed through the light-harvesting complex is in excess of that which can be dissipated or transduced by photosystem II (PSII). This imbalance [excess excitation energy (EEE)] can be generated by excess light (EL) or chilling or both and can be strongly enhanced by a combination with other factors such as rapid and large increases in temperature and limitations in nutritional and H_2O status (*1–8*).

Dissipation of such EEE is an immediate response that occurs through heat irradiation (*2, 3*). However, prolonged exposure to the conditions that cause EEE can result in an increase in the generation of reactive oxygen species (ROS) such as singlet oxygen, superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2) (*4, 8*). If the accumulation of ROS under conditions of EEE exceeds the capacity of antioxidant systems to remove them, irreversible photooxidative damage to the chloroplast and the cell may occur. Thus, overproduction of ROS under EEE conditions can ultimately result in the permanent photodamage of leaf tissues (Fig. 1A).

In our experimental system, EEE was generated by EL applied to LL-adapted *Arabidopsis* plants, resulting in the induction of antioxidant defense genes (as *APX2*) (*8, 9*). Leaves from transgenic *Arabidopsis* plants harboring an *APX2-LUC* fusion (*10*) had no detectable luciferase activity when grown under LL conditions, but after challenging with EL, luciferase activity, which could be imaged, was induced (*11*) (Fig. 1B). The induction of the *APX2-LUC* transgene mirrored the induction of the native *APX2* gene in the same plants, as determined by Northern (RNA) blotting (Fig. 1C) (*8*), and therefore could be used as a measure of activation of *APX2* expression. After 2 hours of exposure to EL, the leaves suffered photodamage and lost *APX2-LUC* and *APX2* expression over most of the leaf area (Fig. 1, A through C).

Induction of *APX2-LUC* by EL could be diminished by infiltrating leaves with catalase but could not be diminished with superoxide dismutase (Fig. 2A). The effect of the catalase treatment occurred in a dose-dependent manner (*12*). The vacuum infiltration and incubation procedures did not affect either luciferase activity or the uptake of luciferin in control *APX1-LUC* transgenic lines that expressed this gene under LL conditions (*8, 12, 13*). Thus, H_2O_2 (but not O_2^-) could be involved in the EL-induced expression of *APX2*. Unlike EL, H_2O_2 alone did not induce the expression of *APX2-LUC* sufficiently to be imaged (Fig. 2A), but the expression could be detected by the more sensitive in vitro assay (Table 1) (*11*).

Treatment of leaves with H_2O_2 and then with exposure to EL produced a lesser degree of photooxidative stress and caused a smaller induction of luciferase activity than in the H_2O-treated controls (Fig. 2, B and C, and Table 1). Detached leaves that were pretreated with H_2O_2 and exposed to EL showed a slower decline in F_v/F_m (F_v, variable fluorescence of chlorophyll a; F_m, maximal fluorescence of chlorophyll a) and a smaller decrease in photochemical quenching (q_p) than H_2O-treated control leaves showed (Fig. 2B and Table 1) (*14*), and the H_2O_2-treated leaves did not develop visible photodamage of leaf tissue (Fig. 2C). Similarly, treatment of maize seedlings and potato nodal explants with H_2O_2 has been shown to protect against chilling in the dark and heat stress, respectively (*15, 16*). Our data show that H_2O_2 is involved in the acclimation to conditions evoked by EEE.

Treatment of detached leaves (*12*) with the photosynthetic electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) before exposure to EL blocked the induction of luciferase activity (*8*). *APX2-LUC* was induced in LL by 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB) treatment (Table 1) (*8*). H_2O_2 did not relieve this effect of DCMU on the EL-mediated induction of *APX2-LUC*. Furthermore, the inductive effect of H_2O_2 observed in LL was also blocked by DCMU (Table 1). Thus, redox changes in electron transport through quinone B (Q_B) or plastoquinone (PQ) or both could be essential for the induction of *APX2* (*8, 17–19*) (Table 1).

The above data (Fig. 2, A through C, and Table 1) suggested that H_2O_2 could act as a

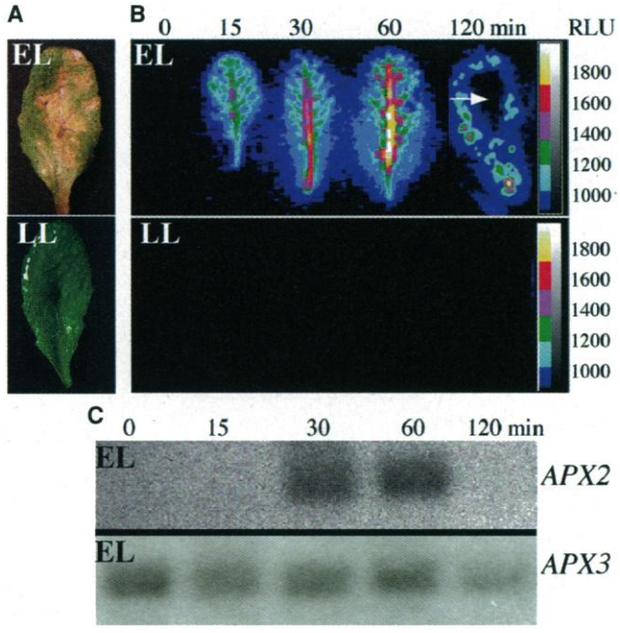

Fig. 1. Permanent photodamage and induction of *APX2-LUC* and *APX2* in transgenic *Arabidopsis* leaf tissue. Leaves of transgenic plant grown in LL (control) were exposed to EL (*9*). (**A**) Appearance of chlorosis on detached leaves after 2 hours in EL. (**B**) CCD camera image of relative luciferase activity (in RLUs) in detached leaves that were exposed to different times of EL (arrow indicates the chlorosis zone of the leaf). (**C**) Gel blot analysis of mRNA levels for *APX2* and *APX3* in leaves that were exposed to different durations of EL (*APX3* mRNA is shown as a loading control).

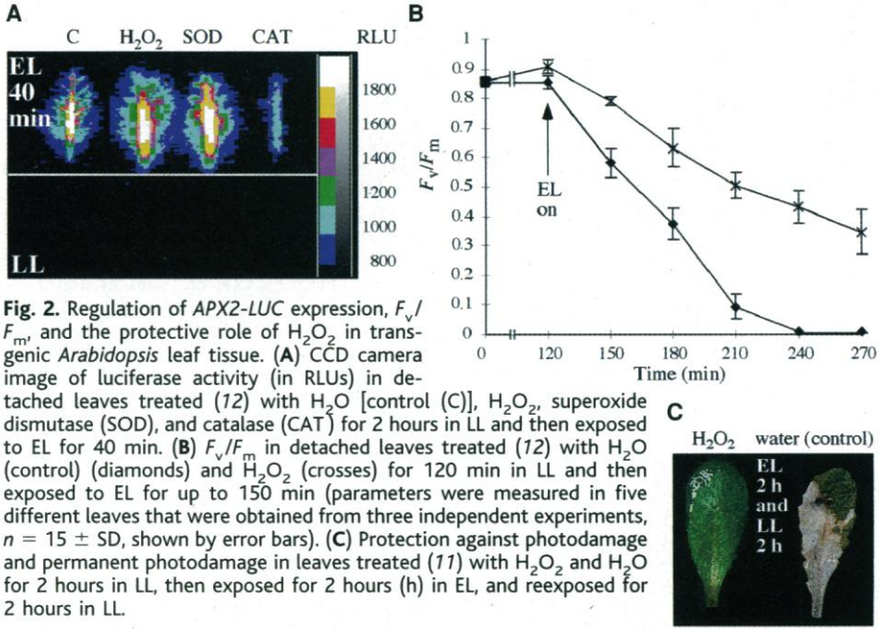

Fig. 2. Regulation of *APX2-LUC* expression, F_v/F_m, and the protective role of H_2O_2 in transgenic *Arabidopsis* leaf tissue. (**A**) CCD camera image of luciferase activity (in RLUs) in detached leaves treated (*12*) with H_2O [control (C)], H_2O_2, superoxide dismutase (SOD), and catalase (CAT) for 2 hours in LL and then exposed to EL for 40 min. (**B**) F_v/F_m in detached leaves treated (*12*) with H_2O (control) (diamonds) and H_2O_2 (crosses) for 120 min in LL and then exposed to EL for up to 150 min (parameters were measured in five different leaves that were obtained from three independent experiments, $n = 15 \pm$ SD, shown by error bars). (**C**) Protection against photodamage and permanent photodamage in leaves treated (*11*) with H_2O_2 and H_2O for 2 hours in LL, then exposed for 2 hours (h) in EL, and reexposed for 2 hours in LL.

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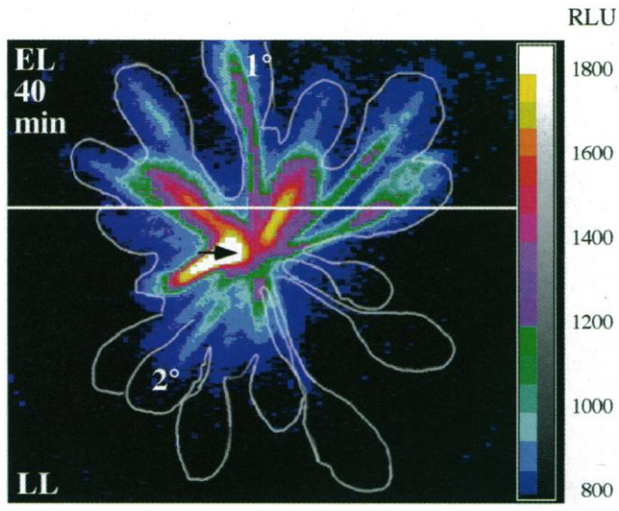

Fig. 3. Systemic induction of *APX2-LUC* expression in transgenic *Arabidopsis* leaf tissue. Image of luciferase activity (in RLUs) (*11*). A part of the whole rosette (as shown) was exposed to EL for 40 min (the arrow indicates the apical region of the rosette). A typical primary (1°) EL-exposed leaf and a secondary (2°) LL-exposed leaf are shown.

Table 1. Role of photosynthetic electron transport and H_2O_2 in the regulation of *APX2-LUC* expression in transgenic *Arabidopsis* leaf tissue. Detached leaves were treated in LL with H_2O (control), H_2O_2 (10 mM), DBMIB (12 μM), DCMU (10 μM), or DCMU and H_2O_2 and exposed to EL for 1 hour (*9–12*). Luciferase activity was expressed as RLUs per gram of fresh weight. All parameters were measured in five different leaves obtained from three independent experiments (n = 15 ± SD). All treatments were statistically tested against the control. Levels of significance were calculated from the analysis of variance (ANOVA) data. ΦPSII, quantum yield of PS II electron transport; LL → EL, subsequent exposure to EL after LL treatments.

Treatment	LL (up to 2 hours)			LL → EL (1 hour)		
	ΦPSII	q_p	Luciferase activity	ΦPSII	q_p	Luciferase activity
H_2O	0.68 ± 0.03	0.85 ± 0.02	10 ± 10	0.16 ± 0.04	0.5 ± 0.05	6413 ± 534
H_2O_2	0.78 ± 0.04*	0.9 ± 0.03**	532 ± 127*	0.34 ± 0.06*	0.74 ± 0.05*	5054 ± 623*
DBMIB	0.44 ± 0.07*	0.57 ± 0.08*	3285 ± 837*	0.21 ± 0.08	0.97 ± 0.22*	3341 ± 1459*
DCMU	0.09 ± 0.06*	0.11 ± 0.07*	27 ± 18	0.01 ± 0.01*	0.03 ± 0.03*	57 ± 49*
DCMU and H_2O_2	0.18 ± 0.05*	0.28 ± 0.06*	84 ± 25*	0.04 ± 0.04*	0.08 ± 0.05*	56 ± 31*

*P < 0.01. **P < 0.05.

Table 2. Systemic acquired acclimation to EL (*9*) of *APX2-LUC* transgenic *Arabidopsis* leaf tissue. Photosynthetic parameters, H_2O_2 content (in micromoles per gram of fresh weight), luciferase activity (in RLUs per gram of fresh weight), and mRNA levels [in relative units (RUs)] (*APX2* mRNA level after 30 min in EL is 1; *APX3* mRNA level in LL is 1) after different light treatments. Treatment A, control rosette exposed to LL; treatment B, rosette partially exposed (~30%) to EL for 30 min (1°, EL-exposed leaf; 2°, LL-exposed leaf); treatment C, rosette partially exposed to EL for 30 min, which was then completely exposed to a second EL treatment for another 30 min (1°, EL and EL–exposed leaf; 2°, LL and EL–exposed leaf). Parameters were measured in five different leaves that were obtained from three independent experiments (n = 15 ± SD). In RNA slot blot experiments, pooled samples of 15 leaves from three independent experiments were used. Treatments B1° and B2° were statistically tested against treatment A; treatments C1° and C2° were tested against B1° and B2°, respectively. Levels of significance were calculated from the ANOVA data.

Treatment	Leaf	F_v/F_m	q_p	H_2O_2	Luciferase activity	mRNA levels	
						APX2	*APX3*
A	LL	0.84 ± 0.02	0.85 ± 0.02	4.2 ± 0.8	10 ± 10	0.0	1.0
B	1°	0.62 ± 0.04*	0.7 ± 0.03*	6.8 ± 0.7*	6747 ± 589*	1.0	1.3
	2°	0.78 ± 0.03*	0.8 ± 0.04	5.6 ± 0.5*	780 ± 238*	0.1	1.1
C	1°	0.4 ± 0.08*	0.5 ± 0.06*	7.3 ± 0.7	6282 ± 421	1.0	0.9
	2°	0.72 ± 0.04	0.78 ± 0.03	5.9 ± 0.6	4728 ± 443*	0.5	1.1

*P < 0.01.

systemic messenger. To test this, we exposed leaves on approximately one-third of the rosette to EL for 30 min (hereafter called 1° leaves), and we measured leaves from the LL side of the rosette (hereafter called 2° leaves) (Fig. 3 and Table 2). *APX2* expression in the 2° leaves was induced to ~11% of the levels in EL-exposed 1° leaves, which was similar to that observed in H_2O_2-treated leaves (Table 1). This activation of *APX2* expression in 2° leaves was associated with an increase in H_2O_2 content and changes in F_v/F_m (Table 2). Under these conditions, 1° leaves showed clear signs of photooxidative stress. Subsequently, a full exposure of partial EL-treated rosettes to further EL for 30 min exacerbated these stresses in 1° leaves, but 2° leaves showed acclimation to EEE and photooxidative stress. They displayed only a slight reduction in F_v/F_m, a slight decrease in q_p, no further increase in H_2O_2 amounts, and less *APX2* induction than that in 1° leaves (Table 2). These data indicate that a systemic signal can control an acclimatory response to EEE.

Our work allows a unified view of acclimatory responses to any fluctuating environmental condition that elicits EEE. When a leaf experiences a set of conditions such as EL, an induction of antioxidant defenses is one of the many cellular responses and is controlled at least in part by the redox status of the Q_B or PQ pool or both (*8, 17–19*) (Table 1). However, cells suffering these stresses also produce a systemic signal, a component of which is H_2O_2, which sets up an acclimatory response to EEE and, consequently, a photooxidative stress in unstressed regions of the plant (Table 2). Furthermore, because changes in the photosynthetic parameters have been observed in 2° leaves (Table 2), we suggest that a systemic signal can promote redox changes in the proximity of PSII in unstressed chloroplasts (Table 1), thus inducing protective mechanisms in remote chloroplasts and cells. We have termed this phenomenon "systemic acquired acclimation."

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following reaction: 2 ascorbate + H_2O_2 → 2 monodehydroascorbate + 2 H_2O.

9. The transgenic plants were grown and exposed to EL as described (*8*) with the following modifications: The photoperiod was 8 hours at 200 ± 45 μmol of photons m^{-2} s^{-1}, night temperature was 16° ± 1°C, EL exposure was 2700 ± 300 μmol of photons m^{-2} s^{-1}. Six- to eight-week-old plants were used in these experiments. EL was generated with a HMV 1200 lamp (Pani, Vienna). Chlorophyll a fluorescence parameters were determined with a portable modulating fluorimeter FMS1 and the manufacturer's software (Hansatech, Kings Lynn, UK).
10. The *APX2* promoter was cloned by polymerase chain reaction (PCR) amplification from the bacteriophage λ clone of *APX2* [M. Santos *et al.*, *Planta* **198**, 64 (1996)] with the following primers: 5′-CCAAGAAGAGGAAAACCGGTACCAAGGTAATCTCAATACTTG-3′ (top strand; coordinates are −1438 to −1397, counted back from the first base of the *APX2* start codon) and 5′-CCGGGTAACTCTTCTTCACCATGGTTTTCAAATTCGCTTCCTTC-3′ (bottom strand, coordinates are +22 to −22). The underlined residues G and GG were changes introduced from the wild-type sequence (C and AA, respectively). These sites introduced a Kpn I and a Nco I site at the 5′ and 3′ ends of the promoter fragment, respectively. The PCR fragment was cut with these enzymes and cloned into pJIT163 [F. Guerineau, A. Lucy, P. Mullineaux, *Plant Mol. Biol.* **18**, 815 (1990)], substituting the cauliflower mosaic virus 35S promoter with the *APX2* promoter. The promoter region was completely sequenced and was confirmed to be a faithful copy of the gene sequence, apart from the introduced changes. The modified firefly luciferase coding sequence cassette (LUC+, Promega, Madison, WI) was inserted as a Nco I–Eco RI fragment, thus creating an *APX2-LUC-CaMV* polyA chimeric gene fusion. This chimeric gene fusion was inserted as a Kpn I–Bgl II fragment into the Kpn I–Bam HI sites of the binary Ti plasmid pBIN19 [M. Bevan, *Nucleic Acids Res.* **12**, 8711 (1984)]. *Arabidopsis thaliana* ecotype Columbia was transformed with an *Agrobacterium*-based procedure [M. Valvekens, M. Van Montagu, M. van Lusebettens, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5536 (1988)]. All of the data in this report were derived from T_4 generation homozygous progeny for one transgenic line. Thus, we have been careful to cross-check all luciferase data by Northern blotting or by RNA slot blotting to examine the response of the native *APX2* gene. RNA isolations, Northern, and slot blot hybridizations were made as described (*8*). We have also performed key experiments on the wild-type plant, which confirm the observed phenomenon.
11. Luciferase activity was imaged with a Berthold luminograph LB 980 charge-coupled device (CCD) camera (Berthold Instruments, Milton Keynes, UK) and processed with the associated software according to the manufacturer's instructions, using an aperture setting of 1.8. Rosettes or detached leaves were misted with 1 mM D-(-)-luciferin (Sigma) until they were uniformly wetted, and then they were incubated for 10 min under LL conditions before imaging. Luciferase activity was also assayed in vitro with a luciferase assay kit (Promega) according to the manufacturer's instructions and measured on a luminometer, model 1250 (BioOrbit, Helsinki). Luciferase activity was expressed as relative light units (RLUs) per gram of fresh weight.
12. Detached leaves were vacuum infiltrated for 3 min in the presence of the various compounds or enzymes dissolved in H_2O or appropriate buffers, respectively. The leaves were then floated on a solution of the same compound or enzyme for up to 2 hours in petri dishes, with the cut end of the petiole submerged below the surface of the liquid, and incubated in LL conditions. After this period, the leaves were exposed to EL. In pilot experiments, the following treatments were done, and the minimum concentration that gave the maximum effect for test parameters was determined: (i) H_2O_2 (0.5 to 100 mM). Ten millimolar H_2O_2-treated leaves gave the highest luciferase activity under LL. (ii) Catalase (bovine) (Sigma) (10 to 200 units per milliliter for 2 hours). Maximum inhibition of EL-induced luciferase activity was achieved with 200 units per milliliter. (iii) Superoxide dismutase (bovine) (Sigma) (10 to 200 units per milliliter). No effect was observed at the highest concentration after 40 min in EL. (iv) DCMU and DBMIB (1 to 20 μM). After 1 hour of incubation in LL, 10 μM DCMU-treated leaves achieved a target q_p value of ≤0.2, and 12 μM DBMIB-treated leaves achieved the highest induction of *APX2-LUC* in LL and a 30% reduction of q_p. Two millimolar stock solutions of DCMU in ethanol and DBMIB in dimethyl sulfoxide were used. In all cases, the q_p value was calculated with measurements made at 300 μmol of photons m^{-2} s^{-1} of actinic light. For the F_v/F_m parameter, leaves were adapted to the dark for a minimum of 30 min before taking the measurement. In combined DCMU and H_2O_2 treatments, leaves were first treated with DCMU and then with H_2O_2. H_2O_2 amounts were determined as described before (*8*).
13. For the construction of *APX1-LUC* transgenic plants, the *APX1* promoter was recovered from pGUS1 (a gift from D. Inzé, University of Gent, Gent, Belgium). pGUS1 harbors a 1.55-kb portion of the *Arabidopsis APX1* immediately upstream of the translation initiation codon [G. H. Kubo, H. Saji, K. Tanaka, N. Kondo, *FEBS Lett.* **315**, 313 (1992)]. This portion was recovered as a Hind III–Nco I fragment and inserted into the same sites of pNonedescript [A. Edwards *et al.*, *Plant Physiol.* **112**, 89 (1996)]. This insert could then be recovered as a Kpn I–Nco I fragment and was inserted into the corresponding sites of *APX2-LUC* (*10*), thus replacing the *APX2* promoter with the *APX1* promoter. The rest of the manipulation steps and transformation were as in (*10*). More than 10 independent *APX1-LUC* transgenic lines were recovered, and all displayed high luciferase activity in LL.
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Rbx1, a Component of the VHL Tumor Suppressor Complex and SCF Ubiquitin Ligase

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The von Hippel–Lindau (VHL) tumor suppressor gene is mutated in most human kidney cancers. The VHL protein is part of a complex that includes Elongin B, Elongin C, and Cullin-2, proteins associated with transcriptional elongation and ubiquitination. Here it is shown that the endogenous VHL complex in rat liver also includes Rbx1, an evolutionarily conserved protein that contains a RING-H2 fingerlike motif and that interacts with Cullins. The yeast homolog of Rbx1 is a subunit and potent activator of the Cdc53-containing SCF^{Cdc4} ubiquitin ligase required for ubiquitination of the cyclin-dependent kinase inhibitor Sic1 and for the G_1 to S cell cycle transition. These findings provide a further link between VHL and the cellular ubiquitination machinery.

The VHL tumor suppressor gene on chromosome 3p25.5 is mutated in most sporadic clear cell renal carcinomas and in VHL disease, an autosomal dominant familial cancer syndrome that predisposes affected individuals to a variety of tumors (*1*). The VHL protein is expressed in most tissues and cell types and appears to perform multiple functions, including repression of hypoxia-inducible genes (*2*), regulation of p27 protein stability (*3*), and regulation of fibronectin matrix assembly (*4*). VHL is found in a multiprotein complex with Elongin B, which is ubiquitin-like, and Elongin C and CUL2, which share sequence similarity with the Skp1 and Cdc53 components of the SCF ubiquitin ligase (*5*, *6*). The Elongin BC complex interacts with a short BC-box motif in VHL and bridges its interaction with CUL2 (*6*). A large fraction of VHL mutations alter the BC-box and disrupt the VHL complex (*1*, *4*, *6*).

To investigate VHL function, we purified the endogenous VHL complex from rat liver (Fig. 1) (*7*). Greater than 90% of the detectable VHL protein copurified with CUL2, Elongins B and C, and a polypeptide of ~16 kD (Fig. 1, B and C). The identities of the VHL, CUL2, and Elongin B and C polypeptides were confirmed by immunoblotting, peptide sequencing, or both. Ion trap mass