Arabidopsis SGT1b Is Required for Defense Signaling Conferred by Several Downy Mildew Resistance Genes

Mahmut Tör,[a] Pam Gordon,[a] Alayne Cuzick,[a,1] Thomas Eulgem,[b] Evaggelia Sinapidou,[a,2] Figen Mert-Türk,[a,3] Canan Can,[c] Jeffery L. Dangl,[b,d] and Eric B. Holub[a,4]

[a] Plant Genetics and Biotechnology Department, Horticulture Research International, Wellesbourne, Warwick CV35 9EF, United Kingdom

[b] Department of Biology, Coker Hall, University of North Carolina, Chapel Hill, North Carolina 27599-3280

[c] Department of Biological Sciences, Gaziantep University, Gaziantep 27310, Turkey

[d] Department of Microbiology and Immunology, Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, North Carolina 27599-3280

We describe the identification of a mutant in the Arabidopsis accession Columbia (Col-0) that exhibits enhanced downy mildew (*edm1*) susceptibility to several *Peronospora parasitica* isolates, including the *RPP7*-diagnostic isolate Hiks1. The mutation was mapped to chromosome IV and characterized physically as a 35-kb deletion spanning seven genes. One of these genes complemented the mutant to full wild-type resistance against all of the Peronospora isolates tested. This gene (*AtSGT1b*) encodes a predicted protein of 39.8 kD and is an Arabidopsis ortholog of yeast SGT1, which was described originally as a key regulatory protein in centromere function and ubiquitin-mediated proteolysis. AtSGT1b contains three tetratricopeptide repeats at the N terminus followed by a bipartite chord-containing SGT domain and an SGT-specific domain at the C terminus. We discuss the role of AtSGT1b in disease resistance and its possible involvement in ubiquitin-mediated proteolysis in plants.

INTRODUCTION

Resistance of plants to biotrophic pathogens is controlled by a complex regulatory system with many features that suggest an ancient origin (for reviews, see Dangl and Jones, 2001; Holub, 2001). Specific molecular recognition of pathogen avirulence (*Avr*) determinants by receptor-like plant proteins, encoded by resistance (*R*) genes, triggers signal transduction processes that activate a variety of defense reactions in infected plants. These inducible defense responses include an oxidative burst resulting from the generation of highly reactive oxygen intermediates (Lamb and Dixon, 1997; Torres et al., 2001), irreversible membrane damage (Woods et al., 1988), hypersensitive death of host cells (Lam et al., 1999), increased expression of defense-associated genes (Maleck et al., 2000; Schenk et al., 2000), and synthesis of antimicrobial metabolites such as phytoalexins (Glazebrook et al., 1997).

Two key defense regulators that are required for the function of multiple *R* genes have been identified in Arabidopsis: *NDR1* (Century et al., 1995) encodes a potentially membrane-associated protein of unknown function (Century et al., 1997), and *EDS1* (Parker et al., 1996) encodes a soluble protein that has homology with eukaryotic lipases (Falk et al., 1999). Signaling through both EDS1 and NDR1 activates a common set of defense responses, including the synthesis of salicylic acid, an important component in local and systemic disease resistance (Feys and Parker, 2000).

Many *R* genes have been shown to preferentially use either *NDR1* or *EDS1* to confer resistance against either bacterial or eukaryotic pathogens in Arabidopsis (Aarts et al., 1998). Most of the *R* genes known at the time from Arabidopsis were used in this analysis, all of which encode receptor-like protein products that contain a carboxyl Leu-rich repeat domain (LRR) and a central nucleotide binding site

(NB) (referred to hence as NB-LRR genes). Aarts et al. (1998) observed that *R* gene functional subsets appeared to correlate with different structural subclasses of NB-LRR genes: the *NDR*-dependent subclass encodes proteins with an N-terminal coiled-coil domain (CC), whereas the *EDS1*-dependent subclass encodes proteins with an N terminus that resembles cytoplasmic domains of the Drosophila Toll and mammalian interleukin 1 transmembrane receptors (TIR domain). Therefore, it was proposed that EDS1 and NDR1 relay alternative defense signals that originate from different subclasses of *R* gene products and converge upstream of salicylic acid accumulation.

Several exceptions to this model have been defined subsequently using isolates of *Peronospora parasitica* (downy mildew) and *Erysiphe* spp (powdery mildew). For example, *EDS1* was selected and characterized as a gene required for downy mildew resistance conferred by *RPP5* in cotyledons and adult leaves of the Arabidopsis accession Landsberg *erecta* (Ler-0) (Parker et al., 1997), whereas an allele of *RPP5* in Columbia (Col-0), designated *RPP4* (Tör et al., 1994; Van der Biezen et al., 2002), requires *NDR1* in cotyledons (Century et al., 1995) as well as *EDS1*. *RPP8* (McDowell et al., 1998) encodes a CC-NB-LRR protein, and its function is not affected by a single mutation in *NDR1*. *RPP8*-mediated resistance is suppressed weakly in an *eds1 ndr1* double mutant (McDowell et al., 2000), demonstrating that full wild-type resistance is achieved by the accumulation of distinct defense responses, each contributing partially to resistance and regulated by *NDR1*, *EDS1*, or an as yet unknown component. Finally, the powdery mildew resistance gene *RPW8* encodes a coiled-coil protein that requires *EDS1* but not *NDR1* (Xiao et al., 2001).

Similar to *RPP8*, resistance conferred by *RPP7* (in Col-0 and recognizing isolate Hiks1) was largely unaffected by single mutations in defense regulators, including the two described above, as well as others that affect phytoalexin production or salicylic acid, jasmonic acid, and ethylene signaling (Glazebrook et al., 1997; Warren et al., 1999; McDowell et al., 2000). McDowell and colleagues (2000) described double mutant analysis that suggested that *RPP7*-mediated resistance also was achieved by cumulative defense responses. *RPP7* has yet to be isolated; however, it has been mapped to a 100-kb interval that spans one of the largest groups of NB-LRR genes in Arabidopsis. None of these is in the TIR subclass, but several share high sequence homology with *RPP8* and *RPP13* (Holub, 2001; A. Cuzick and E.B. Holub, unpublished data).

Here, we describe the identification of a 35-kb deletion mutant in Col-0 designated *edm1* (enhanced downy mildew) that largely suppresses *RPP7* resistance to Hiks1 as well as resistance to several additional Col-0–incompatible Peronospora isolates. The deletion spans seven genes, including one that encodes a protein similar to yeast SGT1, which was described by Kitagawa et al. (1999) as a key regulatory protein in centromere function and ubiquitin-mediated proteolysis.

RESULTS

Phenotypic Characterization of Col-*edm1*

The wild-type phenotype of *RPP7*-mediated resistance in Arabidopsis to infection by the Peronospora isolate Hiks1 has been described macroscopically as a barely visible host response (minute, necrotic flecks) that completely restricts parasite reproduction (Holub et al., 1994). Trypan blue staining of whole cotyledons 7 days after inoculation revealed membrane disruption of host cells bordering the penetrating hyphae (McDowell et al., 2000). The resistance is visible microscopically as complete cessation of parasite growth within the first 24 to 48 hr of infection, with the penetrating hyphae terminating soon after producing one or two haustoria in adjacent mesophyll cells (Figure 1A). A rapid host response becomes visible 48 hr after inoculation, as indicated by callose and autofluorescent deposits (probably phenolic compounds) in host cells surrounding the penetrating hyphae. Callose ensheathment of haustoria and host cell collapse occur in later stages of the wild-type defense response (Figures 1B and 1C).

The *edm1* mutant disrupts *RPP7*-mediated resistance significantly and is an example of a single mutant in Col-0 that is susceptible to Hiks1. The mutant was selected from screening fast neutron–treated Col-5 (*glabrous1* mutant of Col-0) with the Peronospora isolate Cala2, which is recognized in Col-0 by *RPP2* (Holub et al., 1994; Tör et al., 1994). Our extensive attempts to select further fast neutron or ethyl methanesulfonate (EMS) mutant alleles of *edm1* using Hiks1 were unsuccessful (see Methods).

Col-*edm1* supported enhanced sporulation after infection with seven Col-0–incompatible Peronospora isolates that are each recognized by different *RPP* genes (Table 1). The level of susceptibility (measured as the amount of sporulation) varied for each isolate, ranging from heavy (>20 sporangiophores per cotyledon; full susceptibility) produced by Cala2 to low (<10 sporangiophores per cotyledon) produced by Wand1 and Wela3. The other four isolates, including Hiks1, produced moderate sporulation (~15 sporangiophores per cotyledon) in the mutant. The morphology of Col-*edm1* is similar to that of the wild type; however, flowering time appears to be delayed by 1 week or more in the mutant relative to the wild type when plants are grown in either short or long days.

Seedlings of wild-type Col-0, Col-*edm1*, and Col-*rpp7.1* (a fully susceptible EMS mutant of *RPP7*) (Table 2) were compared for differences in their capacity to generate H_2O_2 24 hr after inoculation with Hiks1 using 3,3′-diaminobenzidine (DAB) to stain the infected tissue. This compound captures H_2O_2 and forms a reddish-brown polymer at sites of peroxidase activity (Thordal-Christensen et al., 1997), providing a means to detect an oxidative burst in host cells surrounding penetrating hyphae. More than 100 cotyledons from mutant and wild-type seedlings were inspected. DAB staining was observed in two to four cells per infection site
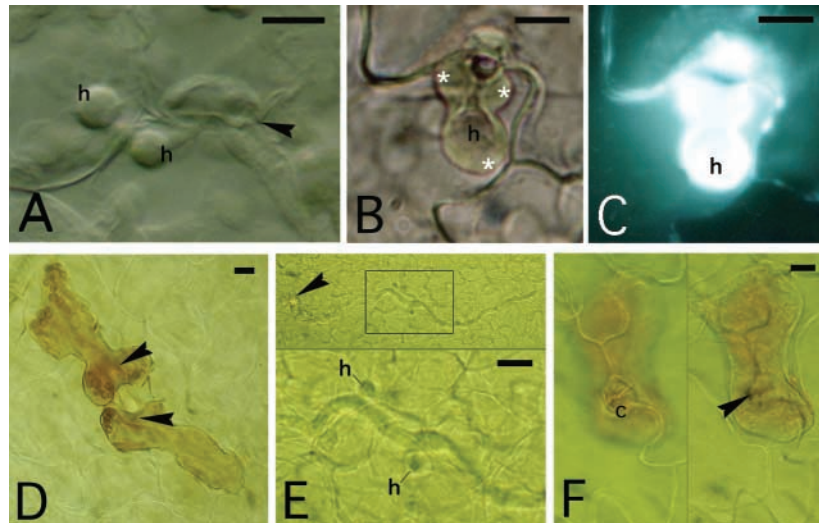
Figure 1. Cotyledons of Wild-Type Col-0, Col-*edm1*, and Col-*edm1*::*SGT1b* Infected with the Peronospora Isolate Hiks1.

(A) Twinned haustoria (h) produced by the parasite in adjacent mesophyll cells before a visible host response in wild-type Col-0 photographed 1 day after inoculation.
(B) Callose ensheathment (white asterisks) of a haustorium in the wild type photographed 7 days after inoculation.
(C) Same as **(B)** but fluorescing under blue light excitation from aniline blue staining of callose.
(D) H_2O_2 accumulation detected with DAB staining 1 day after inoculation in mesophyll cells of the wild type.
(E) No detection of H_2O_2 with DAB staining in Col-*edm1* 1 day after inoculation.
(F) DAB staining 1 day after inoculation of mesophyll cells of Col-*edm1*::*SGT1b*.
Arrowheads indicate penetration sites between anticlinal walls of the epidermal cells (higher focal plane). Bars = 10 μm.

in every wild-type cotyledon, with an average of 10 infection sites per cotyledon. No DAB staining of infection sites was observed in cotyledons of either mutant or in noninoculated control cotyledons. Examples are shown in Figures 1D and 1E. Thus, *edm1* disrupts *RPP7* function upstream of the oxidative burst.

We also tested Col-*edm1* for altered resistance to isolates of the bacterial pathogen *Pseudomonas syringae* strain DC3000 expressing different *Avr* determinants: *avrRpm1* and *avrPphB* eliciting *NDR1*-dependent resistance conferred by *RPM1* and *RPS5*, respectively; and *avrRps4* eliciting *EDS1*-dependent resistance conferred by *RPS4* (Aarts et al., 1998). In all cases, bacterial growth was found to be of similar magnitude in wild-type and *edm1* plants. Growth of *avrRpm1* or *avrPphB* expressing bacteria in *ndr1.1* seedlings and growth of *avrRps4* expressing bacteria in *pad4.1* seedlings were significantly higher (data not shown). Thus, *RPM1*, *RPS4*, and *RPS5* are not influenced by *edm1*.

Genetic Characterization of the *edm1* Mutation

An *rpp7* EMS mutant (Col-*rpp7.1*) was crossed to the *edm1* mutant. The resulting F1 seedlings exhibited wild-type resistance after inoculation with Hiks1 (Table 2), indicating that both mutations were recessive and that the *edm1* mutations

affected a gene(s) other than *RPP7*. This was confirmed using the isolate Cala2 to test F2 seedlings from an outcross between Col-*edm1* and the Cumbrian accession Keswick (Ksk-1); Col-0 and Ksk-1 share *RPP2* alleles that recognize this isolate (Holub et al., 1994). The segregation in this experiment was 107 resistant to 25 susceptible (3:1; χ^2 = 0.65, P = 0.45), suggesting that *edm1* was the only susceptibility determinant segregating in this cross.

For fine-scale mapping of *edm1*, we took advantage of extensive polymorphic loci already available from the Arabidopsis research community between Col-0 and another standard accession, Ler-0 (see Methods). Ler-0 was predicted previously to carry a functional *RPP7* allele because we were unable to select a Hiks1-susceptible recombinant individual from more than 5000 F2 Col-0 × Ler-0 seedlings (McDowell et al., 2000). The F2 Col-*edm1* × Ler-0 segregated in a ratio of 800 resistant to 70 susceptible seedlings after inoculation with Hiks1. We expected ∼217 susceptible seedlings as a result of the recessive *edm1* mutation; thus, our result suggested that Ler-0 carries a second resistance gene (designated *RPP27*) that is *EDM1* independent. We tentatively mapped both loci using 20 Hiks1-susceptible segregants (i.e., homozygous for Col-0 mutant alleles of *edm1* and *rpp27*) from this outcross population (see Methods). The *EDM1* locus then was identified by cross-referencing the closely linked markers (F17A8 and SC5) from one

Table 1. Reproduction in Arabidopsis (Wild-Type Col-0 and Col-*edm1*) of Seven Col-0–Incompatible Isolates of Peronospora (Downy Mildew), Each Recognized by a Different Host Resistance (*RPP*) Determinant

Isolate	RPP Locus[a]	R-Gene Class[b]	Col-0				Col-*edm1*				Col-*rar1*[e] IP	Col-*ndr1*[e] IP
			Mean[c]	SEM	No.	IP[d]	Mean	SEM	No.	IP		
Cala2	RPP2-*IV*	TIR-NB-LRR	0.74	0.23	72	R	19.70	0.17	58	H	wt	wt
Emoy2	RPP4-IV	TIR-NB-LRR	1.86	0.21	74	L2	15.78	0.75	50	M	M	M
Hiks1	RPP7-I	Non-TIR	0.00	0.00	116	N	14.10	0.77	102	M	R	R
Wela3	RPP6-I	Unknown	0.00	0.00	68	N	6.39	0.59	52	L6	L4	L2
Cand5	nd-polygenic	Unknown	0.93	0.29	52	R	15.00	0.70	94	M	H	H
Hind4	nd-II	Unknown	2.18	0.29	66	L2	11.70	0.92	35	M	L10	L4
Wand1	nd-II	Unknown	0.01	0.01	57	R	5.73	0.55	52	L6	H	L2

[a] Recognition of Peronospora. Chromosomes are indicated by roman numerals. nd, not designated.

[b] For review, see Holub (2001).

[c] Mean number of sporangiophores per cotyledon, counted up to a maximum of 20.

[d] IP, interaction phenotype interpreted from quantitative data: N, no sporulation; R, rare sporangiophore (<1 per cotyledon); L, low (1 to 10 sporangiophores, with nearest incremental number indicated); M, medium (11 to 16 sporangiophores); H, heavy (>16 sporangiophores); wt, same as the wild type.

[e] Phenotypes for Hiks1 were taken from *rar1* and *ndr1* controls in Table 2. All other phenotypes were taken from the previous report by Warren et al. (1999). (Note that the *rar1* allele used in this study was referred to as *pbs2*.)

of the loci with the single *edm1* locus that could be mapped in the Col-*edm1* × Ksk-1 outcross population using Cala2.

A total of 310 Hiks1-susceptible F2 Col-*edm1* × Ler-0 seedlings was used to fine map *EDM1* within an interval spanned by F17A8 and SC5 (Figure 2A). New markers were generated from the sequence information of two bacterial artificial chromosomes (BACs) that span the *EDM1* region (T22B4 and F8L21), including markers YRT1 and DAT4, which were used to identify five key recombinant individuals (Figure 2B). Further attempts to generate markers within this interval revealed a 35-kb deletion, determined by the lack of

polymerase chain reaction (PCR) amplification and DNA gel blot hybridization (Figure 3).

An *SGT1*-like Gene Restores Wild-Type Resistance in Col-*edm1*

Transgenic complementation of the *edm1* deletion was performed with seven constructs that contained a single gene from the deleted region (Figure 2C). All of these constructs were used to transform Col-*edm1* plants. T2 seedlings from

Table 2. Genetic Characterization of the *edm1* Mutant of Arabidopsis (Col-0) That Exhibits Enhanced Downy Mildew after Inoculation with Peronospora Isolate Hiks1

Arabidopsis Genotype	Selfed or F1[a]				F2					χ^2	P
	Mean	SEM	No.	IP[c]	N[d]	L	M + H	Observed	Predicted		
Col-0 (*RPP7*)[b]	0.0	0.0	380	N							
Col-*rpp7.1*	20.0	0.0	207	H							
Col-*edm1*	13.7	0.8	274	M							
Col-*ndr1*	0.1	0.1	154	R							
Col-*rar1.21*	0.1	0.1	254	R							
Col-*rpp7.1* × Col-*edm1*	2.5	0.5	48	L2	157	6	144	163:144	173:134 (9:7)	1.3	0.25
Col-*edm1* × Col-0	0.0	0.0	92	N	40	2	12	42:12	41:14 (3:1)	0.2	0.64
Col-*rpp7.1* × Col-0	0.8	0.4	10	L1	28	19	12	38:12	38:12 (3:1)	0.0	1.00

[a] Selfed seed from parents used in crosses (bottom half) or control accessions; F1 seed correspond to segregating F2 populations. Values shown are mean numbers of sporangiophores per cotyledon.

[b] *RPP7* is the *R* gene that confers resistance to Hiks1 in Col-0.

[c] IP, interaction phenotype interpreted from quantitative data: N, no sporulation; R, rare sporangiophore (<1 per cotyledon); L, low (1 to 10 sporangiophores, with nearest incremental number indicated); M, medium (11 to 16 sporangiophores); H, heavy (>16 sporangiophores).

[d] Number of progeny in N, L, M, or H sporulation classes, which was used to calculate the observed resistant (N + L) to susceptible (M + H) ratio.

a minimum of 10 independent transformants were assessed for restoration of resistance after inoculation with Hiks1, Cala2, and Cand5. The construct MTPL, which contains a 4950-bp DNA fragment encompassing an *SGT1*-like gene (At4 g11260), restored wild-type resistance in Col-*edm1* to all three isolates and restored H_2O_2 production (Figure 1F). T2 seedlings from the complementing lines all segregated for the *EDM1* and *edm1* phenotypes (mostly 3:1), correlating completely with Basta resistance and sensitivity, respectively (data not shown).

Sequence and Expression Analysis of SGT1b

Database searches with the *SGT1*-like gene identified several expressed sequence tags (ESTs) that were obtained and resequenced. One of these was confirmed as a full-length cDNA, and comparison of the genomic and cDNA sequences allowed us to define 10 exons and 9 introns (data available from http://mips.gsf.de/). Further database searches identified another *SGT1*-like homolog in Arabidopsis Col-0 located ~30 centimorgan telomeric to At4 g11260 on the BAC clone F9D16. An Arabidopsis EST corresponding to this gene was referred to previously by Shirasu et al. (1999) as Arabidopsis *SGT1*. Hence, we refer to this homolog as *AtSGT1a*, and the gene that complements the *edm1* deletion is designated *AtSGT1b*.

Blast searches demonstrated that *AtSGT1b* has high homology with *AtSGT1a*, a gene from *Rubus idaeus*, a rice *SGT1* gene, and the yeast *SGT1* gene (Figure 4). Overall, *AtSGT1b* shares 72, 64, 60, and 32% amino acid identity, respectively, with the predicted protein products of the other genes. *AtSGT1b* encodes a predicted protein of 358 amino acids (molecular mass of 39.8 kD) that is rich in Glu (12.85%), Ala (12.29%), Lys (11.17%), and Gln (10.61). Hydropathy plot analyses indicated that AtSGT1b is a soluble protein with no obvious transmembrane domains. AtSGT1a, AtSGT1b, and rice SGT1 have three tetratricopeptide repeats (TPRs) (Goebl and Yanagida, 1991) at the N-terminal region. The *R. idaeus* cDNA encodes two copies of TPR repeats, whereas the TPR motif is absent in yeast SGT1. The central region of AtSGT1b shares a bipartite CS domain described by Shirasu et al. (1999) with AtSGT1a, rice SGT1, and *R. idaeus* SGT1 but not with yeast SGT1. An SGT-specific (SGTS) domain described by Kitagawa et al. (1999) includes ~100 amino acids at the C-terminal end in SGT1 proteins from species including those represented in Figure 4 as well as human, *Drosophila melanogaster*, and *Caenorhabditis elegans*.

The presence of several ESTs in the database indicated that *AtSGT1b* is expressed constitutively in noninfected tissue of Arabidopsis. This was confirmed and compared with infected tissue by isolating total RNA from wild-type Col-5, Col-*rpp7.1*, and Col-*edm1* at 0, 6, 12, 24, and 48 hr after inoculation with Hiks1. Reverse transcriptase–mediated (RT) PCR analysis (Figure 3B) was performed using gene-spe-
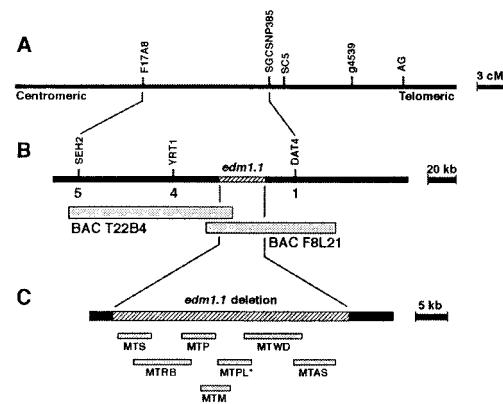

Figure 2. Map-Based Cloning of Arabidopsis SGT1b.

(A) Genetic map of the *edm1* locus indicating molecular markers SC5 and F17A8 that were used initially to define the mapping interval. cM, centimorgan.
(B) The BAC contig spanning the *EDM1* locus, with markers used to define the interval shown above the bar and the number of recombinant individuals with each marker shown below the bar.
(C) Constructs used to complement the Col-*edm1* mutant. The clone that restored wild-type resistance in the mutant is indicated by the asterisk. The constructs each contain a single gene from the deleted region and are as follows: MTS, At4 g11220, which has similarity to a seed maturation protein from soybean; MTRB, At4 g11230, which has strong similarity to the respiratory burst oxidase homolog F; MTP, At4 g11240, a phosphatase type 1 protein; MTM, At4 g11250, a putative protein that may contain an SRF-type MADS box domain; MTPL, At4 g11260, which is described in the text as *AtSGT1b*; MTWD, At4 g11270, which contains two regions with similarity to prokaryotic membrane lipoprotein lipid attachment sites and six WD-40 repeats; and MTAS, At4 g11280, with similarity to 1-aminocyclopropane-1-carboxylic acid synthase.

cific primers. We detected *AtSGT1b* mRNA in Col-5 at 0 hr after inoculation, showing that it is expressed constitutively. Amplification in Col-*rpp7.1* indicated that *AtSGT1b* expression is not *RPP7* dependent. The lack of amplification in the *edm1* deletion mutant demonstrates that the primers used did not detect *AtSGT1a* mRNA and therefore were *AtSGT1b* specific. A time course of *AtSGT1b* expression in Col-5 and Col-*rpp7.1* was very similar at 0, 6, 12, 24, and 48 hr.

DISCUSSION

The striking similarity of three structural domains (TPR, CS, and SGTS) in AtSGT1b to other SGT1 proteins (Figure 4) suggests a highly conserved function of this protein across kingdoms. AtSGT1b has three TPRs at the N-terminal region. The TPR is a degenerate 34–amino acid repeated motif widely reported in a growing number of eukaryotic and
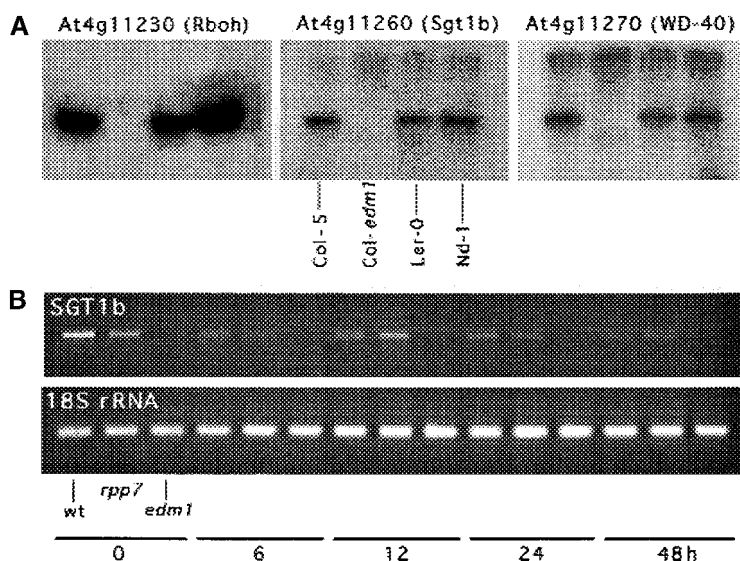
Figure 3. DNA Gel Blot Hybridization of Col-*edm1* Mutant, and Expression Analysis of *SGT1b*.

(A) Genomic DNA from wild-type Col-5, Col-*edm1*, Ler-0, and Nd-1 was digested with BglII, separated by agarose gel electrophoresis, and transferred to a nylon membrane. The membrane was probed with a ^{32}P-labeled PCR product generated from the indicated genes in the locus. Three deleted genes are shown as examples. Gene numbers are given above the panels, and the characteristic feature of each gene is shown in parentheses. Rboh, respiratory burst oxidase homolog F; WD-40, repeat motif of the gene.

(B) Total RNA was isolated from wild-type Col-0 (wt), Col-*rrp7.1*, and Col-*edm1* at 0, 6, 12, 24, and 48 hr after inoculation with Hiks1. RT-PCR analyses were performed with gene-specific primers using 0.5 μg of total RNA as a template. The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. The RT-PCR products were of the expected sizes, and their sequences were identical to that of *AtSGT1b*.

prokaryotic proteins (Lamb et al., 1995). TPR-containing proteins are known to form amphipathic α-helices that mediate protein–protein interaction and have been shown to interact with other TPR or non-TPR proteins (Goebl and Yanagida, 1991; Das et al., 1998). TPR proteins can be involved in a wide array of cellular functions, including cell cycle regulation, transcription control, stress response, protein kinase inhibition, protein transport, Rac-mediated activation of NADPH oxidase, and protein folding (Lamb et al., 1995). In Arabidopsis, a TPR-containing protein, *SPY*, has been shown to be involved in gibberellic acid signal transduction (Jacobsen et al., 1996).

The central bipartite CS domain and C-terminal SGTS domain provide a plausible link between a defense signal relayed from a R–Avr interaction and the SCF complex, which may lead to proteolysis. The CS domain of AtSGT1b is important for interacting with regulatory proteins such as RAR1, which was described originally by Shirasu et al. (1999) in barley. Arabidopsis RAR1 was cloned recently as a defense regulator of several downy mildew and bacterial resistance genes (Muskett et al., 2002; Tornero et al., 2002). The SGTS domain is the most conserved sequence of AtSGT1b, showing high homology with domains in yeast SGT1 and other plant SGT1 proteins (Figure 4). Azevedo et

al. (2002) have reported that both AtSGT1a and AtSGT1b interact directly with Arabidopsis RAR1. In addition, they also have shown that barley SGT1 coimmunoprecipitated with the subunits of the SCF complex, including SKP1, CUL1, and two COP9 subunits (CSN4 and CSN5). Recently, Kitagawa et al. (1999) demonstrated that SGT1 in yeast is required for centromere function and associates with the SCF complex via the SGTS domain. Similarly, the human *SGT1* homolog *SIP* has been shown to interact with the SCF complex for the degradation of β-catenin (Matsuzawa and Reed, 2001).

Null mutations in the single-copy yeast *SGT1* gene are lethal, presumably as a result of its involvement in centromere function (Kitagawa et al., 1999), whereas the deletion of *Atsgt1b* in the Col-*edm1* mutant did not affect viability, presumably because of redundant function provided by AtSGT1a. Therefore, the double mutation of *sgt1a* and *sgt1b* in Arabidopsis may be lethal. If this is the case, examples of disease resistance that rely on both *SGT1* genes may be revealed only by investigating combined mutations of upstream components that are shown to relay independently via *SGT1a* or *SGT1b*. As described below, the *rar1* mutation of Arabidopsis will be useful for such investigations.

A biphasic oxidative burst, resulting from a rapid release of reactive oxygen intermediates (ROI), has been implicated

as an essential component of plant defense signaling during the early stages of pathogen infection (Lamb and Dixon, 1997). The first oxidative burst has been observed as a response to infection in both susceptible and resistant hosts, whereas the second burst appears to correlate only with disease resistance that is typically associated with host–pathogen interactions involving a matching combination of *R* and *avr* gene products (Grant et al., 2000). Localization of ROI in intact plant tissues has been shown with DAB staining, because DAB polymerizes instantly as it comes into contact with H_2O_2 in the presence of peroxidase (Thordal-Christensen et al., 1997). Using this approach, we have shown that H_2O_2 accumulation does not occur in Peronospora-infected tissue of the *edm1* mutant, even though this is a common feature of downy mildew resistance in wild-type Arabidopsis, and that AtSGT1b restored the wild-type phenotype when expressed as a transgene in the mutant.

This finding suggests that AtSGT1b is essential for the production of ROI in defense signaling, acting upstream of ROI production.

Seven isolates of Peronospora that are avirulent in Col-0 can infect and sporulate in the Col-*edm1* mutant. Restoration of wild-type resistance when AtSGT1b was expressed as a transgene in the mutant demonstrates that this gene plays a pivotal role in downy mildew resistance of Arabidopsis. Quantifying parasite reproduction enabled a measurement of residual resistance in the mutant relative to the wild type after inoculation with each isolate. Full susceptibility was observed only in *edm1* with the isolate Cala2 (Table 1), indicating that some degree of alternative (non-SGT1b) defense signaling exists to explain the residual resistance observed with the other isolates. This included *RPP7*-mediated resistance because *edm1* exhibited less sporulation than the control *rpp7* EMS mutant, which was fully susceptible
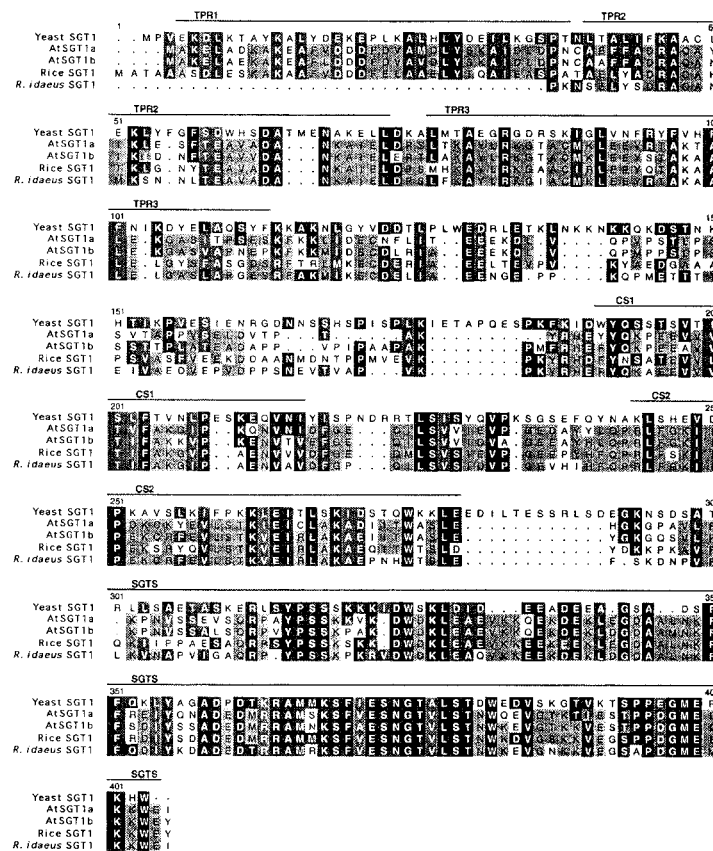

Figure 4. Comparison of SGT1 Amino Acid Sequences from Plants and Yeast.

SGT1-like protein sequences from budding yeast (*Saccharomyces cerevisiae*), Arabidopsis (*AtSGT1a* and *AtSGT1b*), rice (*Oryza sativa*), and *Rubus idaeus* were aligned using Pileup (Wisconsin Package Version 10.0; Genetics Computer Group, Madison, WI). Black or dark gray boxes with white letters indicate identity or similarity to yeast SGT1, respectively. Identity or similarity among the four plant SGT1 sequences exclusively is highlighted in light gray with black letters. The three TPRs (which occur only in the plant sequences), the bipartite CS motifs, and the SGTS are indicated above the sequences.

(Table 2). Alternative defense signaling also was indicated by the discovery of *RPP27* in Ler-0 and from *P. syringae* inoculations in which the different examples of bacterial resistance were affected by the *edm1* mutation. *AtSGT1a* may provide an optional means for some *R* genes to engage with the SCF complex. Otherwise, the alternative resistance probably is achieved via a non-SCF–mediated response.

The full-resistance (no sporulation) phenotype conferred by *RPP7* is similar to examples described in other accessions, including the first example that was described in Rld-0 (Koch and Slusarenko, 1990) and eventually associated with a CC-NB-LRR allele of *RPP13* (Bittner-Eddy et al., 2000). Other similar examples, *RPP5* and *RPP8*, which are TIR-NB-LRR and CC-NB-LRR genes, respectively, were characterized in the accession Ler-0 (Parker et al., 1997; McDowell et al., 1998). Defense signaling conferred by *RPP5* appears to be regulated simply by *EDS1* (Aarts et al., 1998), whereas single mutations (apart from *R* gene loss-of-function alleles) that cause moderate to full suppression of *RPP7*, *RPP8*, or *RPP13* resistance have not been described previously (McDowell et al., 2000; Bittner-Eddy and Beynon, 2001).

Therefore, the major role of *AtSGT1b* in *RPP7*-mediated resistance provides an important advance in the mutational analysis of defense signaling in plants. This is summarized in Figure 5 by a working model that revises a model that we described previously (McDowell et al., 2000). *RPP2*, *RPP4*, and *RPP7* provided contrasting examples of defense regulation in the previous model, and an important question remained regarding whether a common regulator existed among the examples. In the revised model, *SGT1b* is a major defense regulator in all three examples; *RAR1* and a postulated Gene X explain an intermediate link between signaling from the R gene proteins and SGT1b; and *NDR1* provides an alternative defense signal that could explain the residual resistance observed in the single mutants *sgt1b* and *rar1* on *RPP4*- and *RPP7*-mediated resistance (Table 1). Gene X is postulated between RPP2 and SGT1b, interacting directly with the latter protein, because RAR1 is not required for this resistance. This unknown protein could be highly dissimilar from RAR1 if, for example, it interacts with *SGT1b* at the TPR domain instead of the CS domain of SGT1b. Similarly, Gene X could explain why the *rar1.21* null allele exhibits only a minor impact on *RPP7*-mediated resistance, as indicated by a rare sporulation phenotype (Table 2) and confirmed by microscopy (Tornero et al., 2002). Further mutant screening in a *rar1* background for enhanced susceptibility to Hiks1 will be useful in attempts at selection of Gene X mutants.

Double and triple mutant analysis is required to verify the current model. For example, trypan blue staining of cotyledons indicated that *rar1 ndr1* permits enhanced colonization of tissue compared with either single mutation (McDowell et al., 2000; Tornero et al., 2002). Quantifying sporulation in this double mutant will provide a measure of the relative contribution that Gene X provides to *RPP7*-mediated resistance. Other critical examples for testing the model include
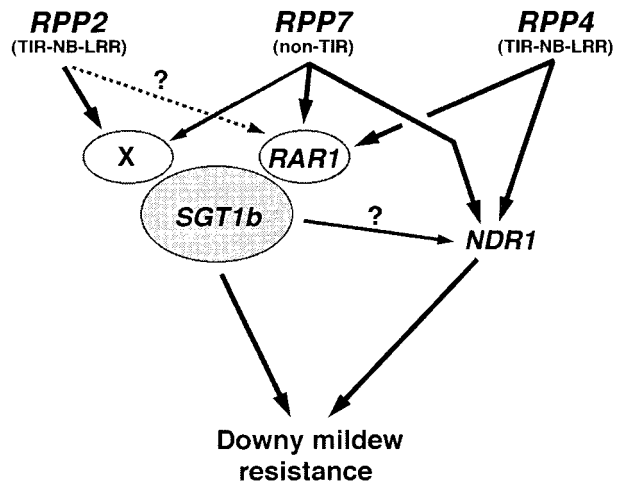

Figure 5. Model Summarizing a Central Role of *AtSGT1b* in *RPP*-Mediated Disease Resistance.

RPP2, *RPP4*, and *RPP7* are three contrasting examples of downy mildew (Peronospora) resistance genes. *NDR1*, *RAR1*, and an unknown defense component (Gene X) may regulate distinct, accumulative defense responses to achieve wild-type resistance. This would explain the residual resistance observed in *rar1* after inoculation with the isolate Cala2 (recognized by *RPP2*) or Hiks1 (recognized by *RPP7*). The model has been modified from the summary of mutant analyses described previously (McDowell et al., 2000).

sgt1b ndr1, which should be fully susceptible to Hiks1 (*RPP7*), and *sgt1b rar1*, which should not differ significantly from *sgt1b* in response to either Hiks1 or Emoy2 (*RPP4*). An alternative method of quantifying the amount of hyphal growth or biomass of Peronospora in host tissue has been developed (Molina et al., 1998) and could be used to compare the quantitative effects of the various mutant combinations in adult leaves.

METHODS

Pathogen Isolates and Pathology Methods

Methods for subculturing *Peronospora parasitica* and preparing inoculum for experiments were modified from Holub et al. (1994). All isolates were maintained in Wassilewskija-*eds1* (Parker et al., 1996), and 10- to 14-day-old seedlings were inoculated (instead of 7-day-old seedlings) to produce large quantities of inoculum. Plants of this age had sufficient height above the soil that enabled clean preparation of spore suspensions with minimal debris. For experiments, 7-day-old seedlings were inoculated as recommended previously. Asexual sporulation was assessed visually 7 days after inoculation by counting low numbers of sporangiophores on both sides of each cotyledon up to a maximum of 10 and recording higher levels as either

medium (~11 to 16 sporangiophores per cotyledon) or high (>16 sporangiophores per cotyledon). Mean sporulation of different host/parasite combinations was calculated using the actual number for low sporulation and 15 and 20 for medium and heavy sporulation, respectively. This method is based on minimal sampling recommended from a rigorous (double-blind) statistical experiment performed by Mert (2001), in which a minimum of 50 cotyledons sampled for a given host interaction ensured significant distinction between no sporulation and rare sporulation, among different levels of low sporulation (e.g., a mean of two versus a mean of six), and between medium and heavy levels of sporulation. *Pseudomonas syringae* experiments were performed as described by Tornero and Dangl (2001).

Arabidopsis Germplasm

Arabidopsis thaliana Columbia-5 (Col-5), Landsberg *erecta* (Ler-0), and Keswick (Ksk-1) used in this study are available from the Nottingham Arabidopsis Stock Centre (http://nasc.nott.ac.uk/). Available mutants were obtained from corresponding authors of previous publications: Col-*ndr1* (Century et al., 1997), Col-*Atrar1.21* (Tornero et al., 2002), and Wassilewskija-*eds1.1* (Parker et al., 1996). Ethyl methanesulfonate (EMS)– and fast neutron–treated M2 seed was purchased from Lehle Seed (Round Rock, TX).

Mutant Screening

Col-*edm1* was selected from a screen of M2 seedlings (derived from ~6000 M1 plants) using the isolate Cala2. The seedlings were sown in 24- × 36-cm trays at a density of ~2000 seedlings per tray and inoculated at 7 days old as described above. We attempted to find additional *edm1* alleles by screening fast neutron– and EMS-treated Col-5 (M2 seed pools from ~6000 and 20,000 M1 plants for each treatment, respectively) with Hiks1 instead of Cala2. No mutant was found that exhibited the same broad-spectrum susceptibility as *edm1*, whereas at least 11 *rpp7* mutants were identified (seven EMS, including *rpp7.1* [described above], and four fast neutron), as well as fast neutron mutations in two additional loci (*EDM2* and *EDM3*; data not shown) that were all fully susceptible to Hiks1 but wild-type resistant to the other isolates.

Light Microscopy

Seedlings infected with Hiks1 and noninoculated controls were removed (severed at the hypocotyl) from soil and placed in Eppendorf tubes containing 100% methanol for 3 to 12 hr. The methanol was replaced with a saturated solution of chloral hydrate to further clear the tissue for 2 to 4 hr. The chloral hydrate was replaced with lactoglycerol (lactic acid:glycerol:water, 1:1:1) for indefinite storage, and seedlings were mounted on glass slides in lactoglycerol for microscopy. For staining, seedlings were placed in different solutions before methanol treatment: aniline blue solution (0.1% [w/v] in 1 M Gly/NaOH buffer, pH 9.5) for 1 to 2 hr to stain callose, and 3,3′-diaminobenzidine stain according to the method recommended by Thordal-Christensen et al. (1997) for the detection of H_2O_2. An Olympus microscope (Tokyo, Japan) equipped with differential interference contrast optics and UV illumination (UV2A and B-3A excitation filters) was used.

Map-Based Cloning of AtSGT1b

We tentatively mapped two loci (*EDM1* and *RPP27*) using genomic DNA from 20 Hiks1-susceptible F2 segregants that were selected from an outcross between Col-*edm1* and Ler-0 (i.e., homozygous for Col-0 alleles of *edm1* and *rpp27*). Cleaved amplified polymorphic sequence markers, which are distributed throughout the genome and polymorphic between Col-0 and Ler-0, were obtained from the Arabidopsis research community (http://arabidopsis.org/aboutcaps.html). One locus was linked closely to g4026 and *RPP7* on chromosome I, and the other was linked to g4539 on chromosome IV. Only Col-5 DNA was detected at both markers in all 20 of these F2 samples (no recombination), whereas markers from other chromosome arms exhibited at least 40% recombination with the Hiks1 susceptibility phenotype. Genomic DNA from 20 Cala2-susceptible F2 Col-*edm1* × Ksk-1 seedlings was used to establish *EDM1* as the locus on chromosome IV. All of these seedlings had inherited Col-0 DNA at marker g4539 (cosegregating with Cala2 susceptibility), whereas g4026 showed 40% recombination. *RPP27*, therefore, was identified as the chromosome I locus; molecular characterization of this resistance gene will be presented elsewhere.

A total of 310 Hiks1-susceptible F2 Col-*edm1* × Ler-0 seedlings were used to fine map *EDM1* within an interval spanned by markers F17A8 and SC5. Sequences of bacterial artificial chromosome (BAC) clones were retrieved, and primers were designed using the software Vector NTI (InforMax, Bethesda, MD) and information from the Cereon Genomics SNP/INDEL database (Cambridge, MA) to generate markers, including SEH2, YRT1, and DAT4. SEH2 was amplified using primers 5′-TGAAACAAGAGAGAAGAATTGAACA-3′ and 5′-TGACAACTAAATCCCTCTTCCTC-3′, and the polymorphism between Col-0 and Ler-0 was detected by the presence of a deletion of ~20 bp. YRT1 was amplified using primers 5′-CATTTTCCCACGGTCTTT-3′ and 5′-TCCTCCTTCACCAAACTCTTG-3′, and the product was cleaved with RsaI to reveal the polymorphism. DAT4 was amplified using primers 5′-TGTAATATCTTTGAAATAAAATGGAGTT-3′ and 5′-ACTTTTTCTCAATTTCATCGTTTGT-3′, and the polymorphism was detected by NdeI restriction digestion. Two overlapping BAC clones (T22B4 and F8L21) that spanned the *EDM1* interval were obtained from the ABRC (Columbus, OH). BAC DNA was prepared using the large construction kit according to the manufacturer's instructions (Qiagen Ltd., UK).

Agrobacterium-Mediated Transformation

Small constructs containing single genes were generated by digesting F8L21 DNA as follows: with NcoI and SalI to release a 4958-bp fragment (At4 g11220); with BspMI to release an 8996-bp fragment (At4 g11230); with SapI to give a 4201-bp fragment (At4 g11240); with PstI to give a 4007-bp fragment (At4 g11250); with NcoI to yield a 4946-bp fragment (At4 g11260); with Eco47III to give an 8638-bp fragment (At4 g11270); and with both Eco47III and BspEI to give a 6260-bp fragment (At4 g11280). The fragments were gel purified, blunt ended using T4 DNA polymerase, and ligated into the SmaI site of the binary vector pCAMBIA3300 (http://www.cambia.org/), which carries the *BAR* gene. Ligated products were electroporated into *Escherichia coli* strain DH10B, and positive clones were identified by polymerase chain reaction (PCR) and restriction analyses. The constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and the *edm1* plants then were transformed with each of the constructs. Transformants were selected by spraying 7-day-old plants grown in soil with 0.04% Basta (AgrEvo,

Norfolk, UK). These selected plants were self-pollinated to produce T2 seed, which were then tested with Hiks1, Cala2, and Cand5 isolates of Peronospora using the methods described above.

Reverse Transcriptase–Mediated PCR and DNA Sequencing

Total RNA was isolated from infected and noninfected cotyledons using the RNAeasy (Qiagen) kit according to the manufacturer's instructions. Single-tube reverse transcriptase–mediated (RT) PCR was performed on 0.5 μg of total RNA using RT-PCR beads (Amersham Pharmacia Biotech). Reactions were performed in a volume of 50 μL using the *SGT1b*-specific primers 5′-ATGGCCAAGGAATTA-GCAGA-3′ and 5′-CTCAATACTCCCACTTCTTGA-3′ by incubating at 42°C for 20 min and denaturing at 95°C for 5 min followed by a standard touchdown PCR regime of 94°C for 30 sec, 65°C for 30 sec (with a 1°C decrement for every cycle), and 72°C for 1 min (10 cycles); and 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min (22 cycles). The 18S primers were used as a control to assess the integrity of RNA. Similar reactions were performed with the 18S primers, but a standard cycling regime of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min (16 cycles) was used. A 5-μL aliquot of each reaction was analyzed by agarose gel electrophoresis.

All DNA sequencing reactions were performed using BigDye Termination Kits (Applied Biosystems, Foster City, CA) and separated on an ABI 377 sequencer. Expressed sequence tag clones and RT-PCR products were sequenced using the universal M13 and the gene-specific primers 5′-ATGGCCAAGGAATTAGCAGA-3′, 5′-CTC-AATACTCCCACTTCTTGA-3′, and 5′-CTGAACATCGGTTTGGCA-GG-3′.

Bioinformatics

Gene sequences from the deleted region were studied using Arabidopsis sequence databases that provide a initial prediction of annotations (TIGR [http://www.tigr.org] and MIPS [http://mips.gsf.de/]). Bioinformatic software used to refine predictions included PIX (http://hgmp.mrc.ac.uk), Interpro (http://www.ebi.ac.uk/), and SMART (http://smart.embl-heidelberg.de/). The sequence of the full-length cDNA corresponding to AtSGT1b was compared with the sequence of the genomic DNA using the alignment program of Vector NTI (InforMax, Inc.), and the coordinates of the exons and introns were confirmed as provided in MIPS.

Accession Numbers

The accession number for the full-length cDNA of the *SGT1*-like gene described in this article is AA712212. The accession numbers for the sequences shown in Figure 4 are NP_014700.1 (budding yeast *SGT1*), CAA23023.1 (Arabidopsis *AtSGT1a*), CAB51410.1 (Arabidopsis *AtSGT1b*), AF192467.1 (rice *SGT1*), and AJ251317.1 (*Rubus idaeus SGT1*).

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