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Murray Grant, Laurence Godiard, Esther Straube, Tom Ashfield, Jürgen Lewald, et al.. Structure of the Arabidopsis RPM1 gene enabling dual specificity disease resistance. *Science*, 1995, 269 (5225), pp.843-846. <10.1126/science.7638602>. <hal-02703241>

**HAL Id: hal-02703241**

**<https://hal.inrae.fr/hal-02703241v1>**

Submitted on 18 Nov 2024

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# Structure of the *Arabidopsis* *RPM1* Gene Enabling Dual Specificity Disease Resistance

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Plants can recognize pathogens through the action of disease resistance (*R*) genes, which confer resistance to pathogens expressing unique corresponding avirulence (*avr*) genes. The molecular basis of this gene-for-gene specificity is unknown. The *Arabidopsis thaliana* *RPM1* gene enables dual specificity to pathogens expressing either of two unrelated *Pseudomonas syringae* *avr* genes. Despite this function, *RPM1* encodes a protein sharing molecular features with recently described single-specificity *R* genes. Surprisingly, *RPM1* is lacking from naturally occurring, disease-susceptible *Arabidopsis* accessions.

Plants express sophisticated genetic systems to recognize pathogens. Complex *R*-gene loci have been defined in a variety of plant-pathogen interactions. Genetic analyses suggest that each *R*-encoded specificity responds to a single *avr*-dependent pathogen signal (1). The recent cloning of four *R* genes (2–7) and accumulation of cloned

bacterial and fungal *avr* genes (8) provide limited insight into the structural basis of specificity, although most models predict that *R* gene products interact with specific pathogen signal molecules produced in an *avr*-dependent manner (9).

*RPM1* was identified in *A. thaliana* accession Col-0 as conferring resistance to *P. syringae* isolates expressing the *avrRpm1* gene (10, 11). Functional homologs of *RPM1* exist in pea, bean, and soybean (12). Resistance in *A. thaliana* to the *P. syringae* *avrB* gene also mapped to the *RPM1* interval (initially termed *RPS3*) (13), and genetic analyses of *A. thaliana* mutants have suggested that *RPM1* conferred resistance to *P. syringae* expressing either *avrRpm1* or *avrB* (14). Because the sequences of *avrB* and *avrRpm1* are unrelated (12, 15), *RPM1* appears to determine a dual specificity.

*RPM1* was mapped by restriction fragment length polymorphism (RFLP) analysis

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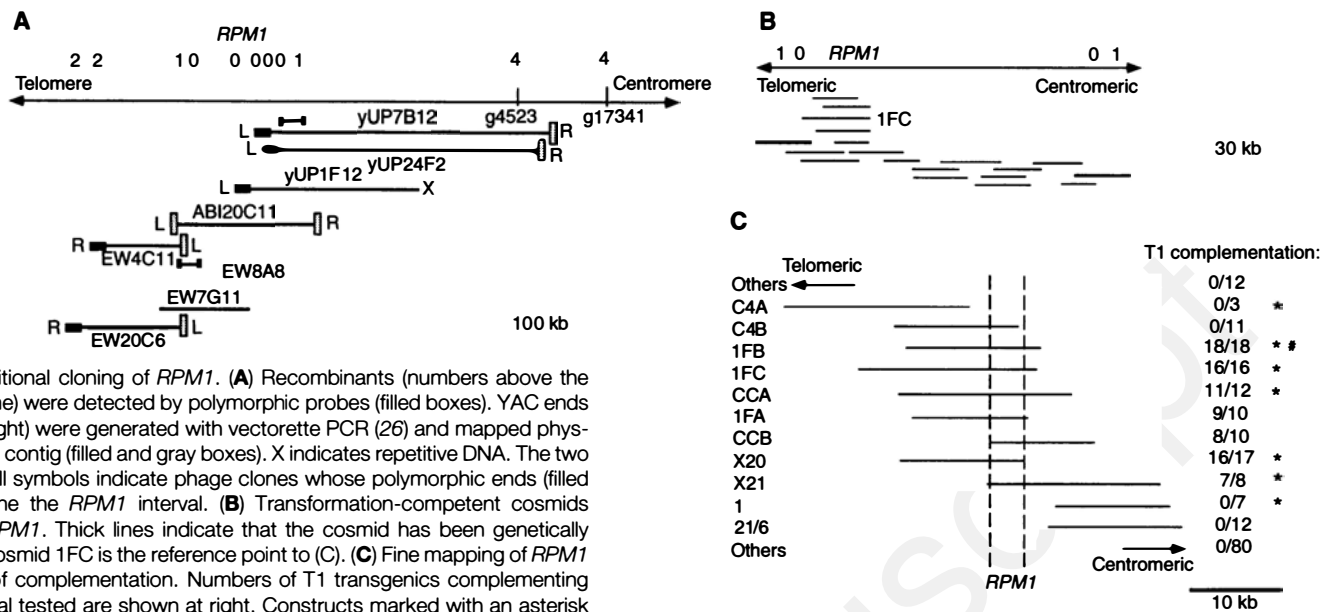
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**Fig. 1.** Positional cloning of *RPM1*. (A) Recombinants (numbers above the chromosome) were detected by polymorphic probes (filled boxes). YAC ends (L, left; R, right) were generated with vectorette PCR (26) and mapped physically on this contig (filled and gray boxes). X indicates repetitive DNA. The two small barbell symbols indicate phage clones whose polymorphic ends (filled boxes) define the *RPM1* interval. (B) Transformation-competent cosmids spanning *RPM1*. Thick lines indicate that the cosmid has been genetically mapped. Cosmid 1FC is the reference point to (C). (C) Fine mapping of *RPM1* by means of complementation. Numbers of T1 transgenics complementing over the total tested are shown at right. Constructs marked with an asterisk were tested in parallel for complementation of resistance to *P. syringae* DC3000 expressing either *avrRpm1* or *avrB*. Also, between two and six T1 individuals from this group were allowed to self-pollinate, and antibiotic-resis-

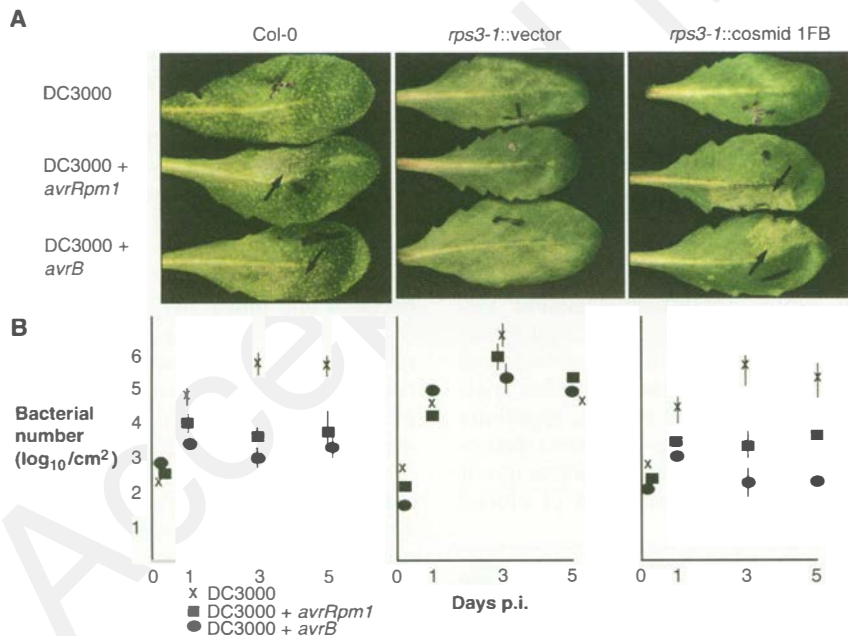
tant T2 progeny (18 per family) were inoculated to confirm results for resistance to both *avr* genes. The number symbol indicates a cosmid that was also used to complement the *rpm1* accession Nd-0.

(11). We assembled an overlapping set of yeast artificial chromosome (YAC) clones covering approximately 800 kb and identified two clones, W8A8 and W7G11 (DNA inserts from accession Col-0 and thus con-

taining *RPM1*), encompassing the *RPM1*-proximal recombination breakpoints. Plant transformation-competent cosmids were isolated from these YACs (16) and organized into a contig (Fig. 1B).

We transformed two loss-of-function *rpm1* mutants (*rpm1-1* and *rps3-1*) (14) and the naturally occurring *rpm1* accession Nd-0 with these cosmids (17). We assessed complementation by inoculating T1 and T2 transgenics with *P. syringae* strain DC3000 [virulent on all *A. thaliana* accessions (18)] expressing *avrRpm1*. For complementation, we expected a hypersensitive response (HR) indicative of *RPM1* action (Fig. 2A) (11, 12). Cosmids CCB and X20 defined a minimal region of 5 kb containing *RPM1* (Fig. 1C). Complementation also rendered plants resistant to DC3000 expressing *avrB* (asterisk, Fig. 1C; Fig. 2A) (11, 12). We quantitated disease resistance by measuring the growth of bacteria in the plants (Fig. 2B). Transformants complementing *rpm1* alleles for triggering of an HR also restricted growth (100-fold) of DC3000 expressing either *avrRpm1* or *avrB* (11, 12). These data show that *RPM1* is confined to a 5-kb region and that this region allows recognition of *P. syringae* expressing either *avrRpm1* or *avrB*.

We identified *RPM1* within the minimum complementing region by sequencing genomic DNA from Col-0 DNA and four mutant *rpm1* alleles (Fig. 3) (19). In 4.5 kb of wild-type DNA, we identified only one large, intronless open reading frame (ORF) of 2778 base pairs (bp) encoding a predicted protein product of 926 amino acids. This predicted mRNA size is in agreement with data from RNA blots (Fig. 4B). A sequence in the expressed sequence tag (EST) database (database accession number T44885) is identical to a



**Fig. 2.** *RPM1* complementation. (A) Restoration of HR. T1 transgenics containing an empty vector or cosmid 1FB, and matched Col-0 controls, were inoculated (arrows) opposite black ink marks with *P. syringae* DC3000 or *avr*-expressing derivatives (listed at left) at  $5 \times 10^7$  colony-forming units (CFU) per milliliter; leaves were photographed 12 hours post-inoculation (p.i.) (11, 12). (B) Complementation inhibits growth of *P. syringae* DC3000 expressing *avrRpm1* or *avrB*. Wild-type Col-0 and *rps3-1* T2 plants transgenic for empty vector or cosmid 1FB were inoculated with *P. syringae* DC3000 or *avr*-expressing derivatives at  $10^5$  CFU/ml. Bacterial growth was monitored over 5 days by collection of leaf punches and titration of bacteria on antibiotic plates selective for markers on the DC3000 chromosome and the plasmid carrying either *avrRpm1* or *avrB* (11, 12). Data points are the means and SD from triplicate determinations. Where no SD is shown, it was smaller than the symbol.

**A**

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1 MASATVDFGI GRILSVLENE TLLLSGVHGE IDKMKKELLI MKSFLEDTHK
51 HGGNGSTTTT TQLFQTFVAN TRDLAYQIED ILDEFGYHIH GYRSCAKIWR
101 AFHFPPRYMNA RHSIAQKLG M VNVMIQSISD SMKRYHYSEN YQAALLPPID
151 DGDAAKVVNNI SESSLFFSEN SLVGIDAPKG KLIGRLLSPE QRIVVAVVG
201 MGGSQKTTLS ANIFKSQSVR RHFESYAWVT ISKSYVIEDV FRMTIKEFYK
251 EADTQIPAE L YSLGYRELVE KLVEYLQSKR YIVVLDVWT TGLWREISIA
301 LPDGIYGSRV NMTTRDMNVA SFPYGIGSTK HEIELLKEDE AWVLFNSKAF
351 PASLEQCRTO NLEPIARKLV ERCOGLPLAI ASLGSMMSTK KFSEWKKVY
401 STLNWELN NN HELKIVRSIM FLSFNDLPYP LKRCFLYCSL FPNVYRMRK
451 RLIRMWMAQR FVEPIRGVKA EEVADSYLNE LVYRNMLQVI LWNPFGRPKA
501 FKMHDVIWEI ALSVSKLERF CDVYNDSDG DDAETMENY GSRHLCIQKE
551 MTPDSIRATN LHSLLVCSA KHKMELLPSL NLLRALDLED SSISKLPDCL
601 VTMFNLYLN LSKTQVKELP KNFHKLVNLE TLNTHKSKIE ELPLGMWKLK
651 KLRYLITFRR NEGHDSNWN Y VLGTRVVPKI WQLKDLQVMD CFNAEDELK
701 NLGCMTQLTR ISLVMVRREH GRDLCDLNLN IKRIRFLSLT SIDEDEPLEI
751 DDLIATASIE KLFLAoKLER VPSWFNTLQN LTYLGLRGSQ LQENAILSQ
801 TLPRLVWLSF YNAYMGPRLR FAQGFQNLKI LEIVQMKHLT EVVIEDGAMF
851 ELQKLYVRAC RGLEYVPRGI ENLNQLQELH LIHVSQNLVE RIRGEGSVDR
901 SRVKHIPAIK HYFRTDNGSF YVLSSS*

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**Fig. 3. (A)** Deduced RPM1 protein sequence (27). Leucine and isoleucines in the putative leucine zipper are in bold on line 1; five other domains discussed in the text are bold and underlined. The LRR region is in italics; proline residues useful in defining LRR units are in bold. Open circles

portion of the RPM1 genomic sequence. We obtained this clone from the Ohio State Arabidopsis Stock Center and found its full sequence to be colinear with the Col-0 genomic sequence, with a polyadenylation sequence beginning 364 bases 3' of RPM1.

The RPM1 ORF (Fig. 3A) contains features found in the predicted polypeptide sequences of other R genes (2-5, 7, 9): a potential six-heptad amphipathic leucine zipper (positions 10 to 51), two motifs of a nucleotide binding site (NBS; positions 200 to 208 and 279 to 288) (20), and 14 imperfect leucine-rich repeats (LRRs) from position 553 (21). These features most closely resemble those of the *A. thaliana* RPS2 gene (23% identity and 51% similarity), which confers resistance to *P. syringae* expressing *avrRpt2* (3, 4). In addition to the NBS and LRRs, three other sequence blocks are shared with RPS2, the tobacco *N* gene, and the flax *L6* gene: a potential kinase 3a site of the NBS (positions 373 to 385) potentially involved in membrane association, and a short domain from positions 431 to 438 (aligned in Fig. 3C). Three potential N-glycosylation sites were found at positions 54, 610, and 917.

We identified mutations in four *rpm1* loss-of-function alleles (Fig. 3B) (14). A nucleotide deletion in codon 818 causes a frame shift and termination after 11 amino acids in *rps3-1*, which suggests a functional requirement for the COOH-terminus of RPM1, including the final three LRR repeat units. The leucine to phenylalanine change in *rps3-2* introduces a bulky aromatic side

**B**

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rps3-1: ΔA, frameshift at R818 to GCVLHKDFRI*
rps3-2: A to T; L301F
rps3-3: T to A; Y87 stop
rps3-4: G to A; G766E

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**C**

	P-loop:	Kinase 2a:	Conserved domain 1 (kinase 3a?):
RPM1	200 GMGGSGKTT	279 KRYIVVLDLV	307 GSRVMMTTRDMNV
RPS2	182 GPGGVGKTT	255 KRFLLLDDV	283 KCKVMFTTRSIAL
N	216 GMGGVGKTT	294 KKVLIIVLDDI	323 GSRIITTRDKHL
L <sup>6</sup>	266 GMGGIGKTT	342 FKILVLDLV	297 QSRFIITSRSMRV

	Conserved domain 2:	Conserved domain 3:
RPM1	373 CQGLPLAIASLGS	431 LKRCFLYC
RPS2	345 CGGLPLALITLGG	404 LRSCFLYC
N	372 AKGLPLALKVWGS	440 DIACFLRG
L <sup>6</sup>	387 TAGLPLTLKVIGS	446 DIACFFIG

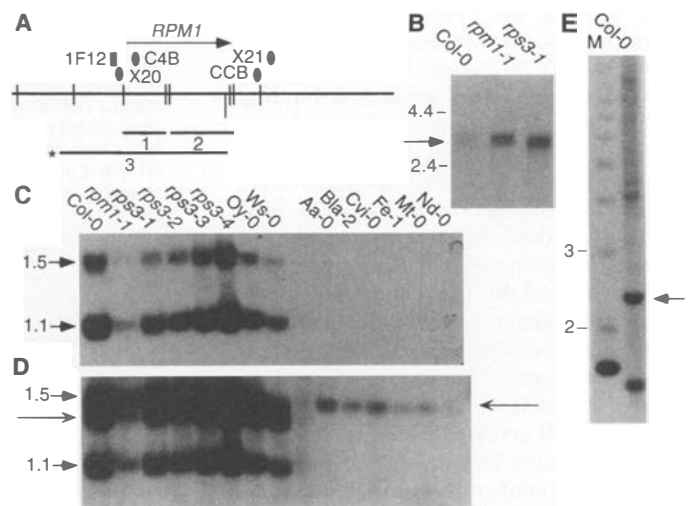
above amino acid residues indicate positions of mutations in the loss-of-function alleles detailed in (B) (27, 28). (C) Alignment of conserved domains among four R genes. Numbers refer to NH<sub>2</sub>-terminal residue position for the respective sequences (27).

chain that may alter juxtaposition of the kinase 2 and kinase 3a domains in the NBS. A glycine to glutamic acid exchange in *rps3-4* introduces a charge and may disrupt the  $\alpha$ -helical structure of an LRR unit (21). Finally, *rps3-3* is a nonsense mutation resulting in termination at codon 87.

Probes internal to the RPM1 ORF (probes 1 and 2, Fig. 4A) detect neither alterations in expression level or message size nor structural changes in DNA from the mutant *rpm1* alleles. RNA blots reveal a rare mRNA of roughly 3.0 kb, which is consistent with the RPM1 ORF size (Fig.

**Fig. 4.** Expression and genomic organization of RPM1. (A) Schematic fine map of RPM1 showing the ORF (arrow), end points of critical cosmids (solid ovals), and the left end of YAC yUP1F12 (solid rectangle) (Fig. 1, A and C). Cosmid names are inside of end-points, and orientation is reversed with respect to Fig. 1. Restriction sites indicated are either Hind III or Xba I (double digestion releases inserts from pCLD05451). Probes 1 and 2 are 1.1-kb and 1.5-kb RPM1 ORF-internal Hind III fragments.

The Xho I site (asterisk) of the 4.5-kb Xho I-Xba I probe 3 is derived from the 1FC cosmid cloning site. (B) RNA blot analysis of RPM1 mRNA (arrow). Polyadenylated RNA was isolated from leaves of wild-type accession Col-0 (2  $\mu$ g) and two mutants (4  $\mu$ g), and blots were hybridized with pooled probes 1 and 2, washed at high stringency (29), and exposed to Kodak X-AR film for 7 days. RNA size standards (in kilobases) were from Gibco-BRL. (C and D) Structure of the RPM1 locus. Blots from Hind III-digested genomic DNA were hybridized with pooled probes 1 and 2 (29); exposure lasted for 30 hours (C). Hybridization was done with probe 3 (D) at high stringency; exposure lasted for 30 hours. The *rpm1-1* and Nd-0 lanes are underloaded. Accessions Col-0, Aa-0, Bla-2, Cvi-0, Fe-1, Mt-0, and Nd-0 are all naturally occurring *rpm1* accessions (18, 20). Thick arrowheads indicate RPM1 gene fragments; thin arrowheads indicate a novel fragment present in natural *rpm1* accessions. (E) Low-stringency blot of Eco RI-digested Col-0 genomic DNA probed with pooled probes 1 and 2 under low-stringency conditions (29) with a 4-hour exposure. Arrow indicates a fragment containing the RPM1 ORF. M is a 1-kb ladder (Gibco-BRL).



4B). Two similarly sized transcripts are produced, as observed for *RPS2* (4) and the tomato *Cf-9 R* gene (6). In contrast, six naturally occurring accessions of *A. thaliana* that are susceptible to infection with *P. syringae* expressing *avrRpm1* or *avrB* lack *RPM1* (Fig. 4C). This is unusual because common features of the *R* locus structure are multigene families and the presence of a homolog or homologs at the corresponding position in susceptible plants (1–7, 9). *RPM1* may be recently evolved or may have been lost in *A. thaliana* accessions through genomic instability at *RPM1*. A DNA probe extending upstream from the *RPM1* ORF (probe 3, Fig. 4A) detected a band of weaker relative intensity in the *rpm1*-null accessions that was roughly 200 bp larger than the corresponding band in *RPM1* accessions (Fig. 4D). All six *rpm1*-null accessions contained the same size band, which suggests that a single event has introduced or deleted *RPM1* from this locus. Low-stringency hybridization revealed one strongly hybridizing and several weakly hybridizing bands that may represent *RPM1*-related sequences elsewhere in the *A. thaliana* genome (Fig. 4E).

Whether *RPM1*, or any *R* gene product, directly interacts with the corresponding *avr*-dependent signal is unknown. The signal produced by *P. syringae* strains expressing either *avrRpm1* or *avrB* could be structurally similar, and the dual specificity of *RPM1* could reflect a single, or overlapping, binding site. The lack of mutants separating these two specificities seems to argue that the *A. thaliana* *RPM1* molecule does not possess two *avr*-signal binding sites. However, genetic analyses of soybean cultivars reveal allelic *R* specificities recognizing *P. syringae* expressing *avrRpm1* or *avrB* or both (22). Alternatively, dual specificity could be a consequence of *RPM1* interacting in a pathway with signals transduced through a promiscuous receptor of *avr*-dependent signals. Plants defend themselves against a variety of pathogens and presumably maintain a large repertoire of functional *R* specificities. The evolution of *R* genes determining multiple specificities may be one way of reducing the absolute number of *R* genes required to meet these demands.

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27. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
28. Four new alleles have been sequenced since submission of this paper: *rps3-5* (EMS-B38) [Gly<sup>384</sup> → Arg<sup>384</sup> (GGA to AGA)]; *rps3-6* (EMS-B61) [Leu<sup>186</sup> → Phe<sup>186</sup> (CTT to TT T)]; *rps3-7* (ENU-B7) [Pro<sup>498</sup> → Ser<sup>498</sup> (CCC to TCC)]; and *rps3-8* (ENU-B8) [(Asn<sup>812</sup> → Ile<sup>812</sup> (AAT to ATT)] (P. Mowery and R. W. Innes, unpublished data). An asterisk in the sequence is a stop.
29. Hybridization was done in 5x standard saline citrate (SSC), 5x Denhardt's solution, 0.1% SDS, and herring sperm DNA (100  $\mu$ g/ml) unless otherwise stated. Membranes (HyBond N, Amersham) were washed in 2x SSC and 0.1% SDS at 65°C for 30 min, then in 1x SSC and 0.1% SDS at 65°C for 30 min. Low-stringency blots were prehybridized and hybridized in 0.5 M Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.2), 1% bovine serum albumin, 1 mM EDTA, and 7% SDS for 16 hours at 55°C. Washings were done twice for 20 min at 50°C in 40 mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.2), 1 mM EDTA, and 5% SDS, followed by two more washes under the same conditions but with only 1% SDS. All other standard techniques were as described in F. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Wiley, New York, 1987) and in J. Sambrook, S. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), vol. 2.
30. We thank C. Lister, J. Jones, and C. Dean for pCLD04541; J. Mulligan and R. Davis for the Col-0 genomic phage library; R. Schmidt for chromosome-walking tips; and D. Boyes for sequence analyses. Supported by grants to J.L.D. from the European Community Human Capital and Mobility Program, the German Federal Ministry of Research and Technology and the German Research Society "Arabidopsis" Focus Program, and by grant R29 GM 46451 from NIH to R.W.I. L.G. received an INRA postdoctoral stipend and T.A. received a North Atlantic Treaty Organization postdoctoral fellowship.