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# Transcriptional Repression Distinguishes Somatic from Germ Cell Lineages in a Plant

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In flowering plants, the male germline begins with an asymmetric division, after which one of the resulting cells, the generative cell, divides symmetrically to produce two sperm cells. We show here that the male germline is initiated by transcriptional control. We identify GRSF, germline-restrictive silencing factor, from the lily. GRSF is ubiquitous in nongerm cells and is absent from male germ cells. GRSF recognizes silencer sequences in promoters of genes specific to the germline, stably repressing these genes in cells that are not destined to become germ cells.

Sexual reproduction in flowering plants requires a pair of sperm that travel together through the pollen tube to the embryo sac. The pair of sperm results from symmetric cell division of the generative cell, which is in turn the result of a preceding asymmetric cell division. Unlike in animals, where the male germline is set aside early in development, in plants the male germ lineage arises from cells of a previously somatic lineage. Some of the gene expression patterns operating in these male germline cells have been identified (*1*, *2*). The genes and proteins that are essential for the unique functions of male germline cells in fertilization are likely to be among those expressed specifically in these cells. Transcripts have been identified that are expressed only in sperm cells or their precursor generative cells (*3–6*). The gene *LILY GENERATIVE CELL-SPECIFIC 1* (*LGC1*) is a male germline–specific gene (*3*, *7*), and its promoter, which contains a silencer region (*7*), can direct the expression of reporter genes in male germline cells of transgenic plants (*7*). We used the *LGC1* gene promoter to study the regulatory mechanisms that control developmental gene expression in the male germline of *Lilium* (lily) and *Arabidopsis*. We found that an essential component of male germline cell–specific regulation of *LGC1* and other coordinately expressed genes lies in a germline restrictive silencing factor (GRSF) that represses their expression in other plant cells.

We prepared a *LGC1* promoter–red fluorescent protein (p*LGC1-RFP*) construct and confirmed its male germline specificity and the presence of a silencer region (fig. S1, A to C). We further reasoned that if in non–male germline cells the *LGC1* promoter is repressed by the binding of a specific repressor to the silencer element, then flooding these cells with an excess of silencer sequence should lead to derepression of the promoter. We designed a competitor comprising 16 ligated repeats of 43–base
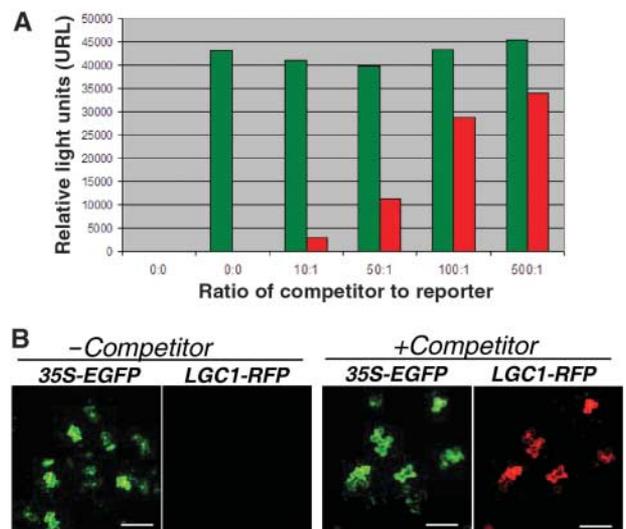
pair (bp) double-stranded oligonucleotide silencer sequences (fig. S1D) and tested it in a transient expression system that involved electroporation-mediated cotransformation of the competitor and p*LGC1-RFP* into lily petal protoplasts (fig. S1E). In the absence of competitor, no expression of RFP was observed. However, cotransformation with competitor led to a reactivation of *LGC1* promoter. We further noticed that increasing the ratio of the competitor versus the reporter construct concomitantly enhanced the level of expression of RFP (Fig. 1A). A p*CaMV35S-EGFP* reporter construct (EGFP, enhanced green fluorescent protein) was also cotransformed in all experiments as an internal control of electroporation efficiency. The presence or absence of competitor had no effect on the expression of GFP under the control of *CaMV35S* promoter. These results revealed that sequestration of repressor by the excess silencer sequences can lead to ectopic activation of *LGC1* promoter. Such competitor-induced ectopic activation of *LGC1* promoter was also observed when p*LGC1-RFP* construct was introduced in lily petal cells

by microprojectile bombardment (Fig. 1B and fig. S1F). These data suggested that the in vivo repression of *LGC1* promoter in non–male germline cells might be mediated by the binding of a sequence-specific repressor.

**LGC1 silencer sequence can recruit silencing machinery to a heterologous promoter.** To investigate whether the *LGC1* silencer sequence is sufficient to recruit transcriptional silencing machinery in vivo, we replaced a 43-bp sequence from a constitutive (*CaMV35S*) promoter (base pairs –216 to –259) with a 43-bp nucleotide sequence from *LGC1* promoter (Fig. 2A). This modified promoter fused with the *EGFP* coding sequence (p*mCaMV-EGFP*) (Fig. 2B) was cotransformed with p*CaMV-RFP* construct into lily petals. As shown in Fig. 2C, RFP signals but not GFP signals were observed in petal cells, indicating that the introduction of silencer sequences from *LGC1* promoter leads to complete inactivation of *CaMV* promoter. However, this modified *CaMV* promoter could be reactivated by cotransformation with an excess of the competitor (Fig. 2D). This activation is attributable to a lack of repressor binding to the silencer region in the modified *CaMV* promoter due to sequestration of repressor by the competitor. This silencing of *CaMV* promoter is sequence specific, because replacement of the same *CaMV* promoter domain with a randomly selected human genome sequence (Fig. 2E) had no noticeable effect on the promoter activity (Fig. 2F). These data show that the silencer sequence is sufficient to recruit a specific repressor and associated transcriptional silencing machinery in the context of a constitutive heterologous promoter.

**Repressor recognition of similar silencer sequences is conserved in flowering plants.** Lily *LGC1* promoter retains its strict generative and sperm cell specificity in a taxonomically distant

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**Fig. 1.** Transcriptional activity of *LGC1* promoter. (**A**) Quantitative fluorometric assay showed that *LGC1* promoter is inactive in lily petal protoplasts, but cotransformation with increasing concentrations of competitor led to concomitant increases in the level of RFP expressed as a result of activation of *LGC1* promoter. In all electroporation experiments, *CaMV35S-EGFP* was cotransformed as an internal control. The amounts of RFP and GFP in biological replicates were quantified with a FLUOstar Optima microplate reader. The plate reader did not detect any signal in electroporated protoplast without the reporter constructs. Green and red bars



denote GFP and RFP levels, respectively. (**B**) Transformation of lily petal cells with mixture of *LGC1-RFP*, *CaMV35S-EGFP*, and competitor led to activation of both *LGC1* and *CaMV35S* promoters, as reflected by the expression of RFP and GFP in the same cell. The expression of GFP as a result of cotransformation with *CaMV35S-EGFP* in all bombardment experiments acted as a positive control. Scale bars, 200 μm.

plant, tobacco (7), implying the conservation of a related sequence-specific repressor common to disparate families of flowering plants. Support for a conserved regulatory mechanism is extended by our further bombardment experiments. We cotransformed petal tissues from taxonomically diverse plants with p*delLGC1-EGFP* constructs, with p*CaMV-RFP* used as an internal control. The *delLGC1* promoter carrying a deletion of the silencer region led to constitutive expression of GFP in petal tissues from all tested plants. These results suggest the presence of functionally conserved repressors in other plants. Further experiments involving cotransformation with p*LGC1-RFP* and silencer competitor led to activation of the *LGC1* promoter in petal cells from such diverse plants as *Brassica*, *Magnolia*, and pea (Fig. 2G), providing evidence for the presence of an evolutionarily conserved repressor in flowering plants.

**Repressor-mediated transcriptional regulation is functional in planta.** *Arabidopsis* plants carrying *LGC1:GUS* construct showed

no β-glucuronidase (GUS) activity (fig. S2A) relative to constitutive expression in plants carrying *del-LGC1-GUS* construct (fig. S2B). Thus, stable transformation provides accompanying in planta evidence that the silencer sequence in the *LGC1* promoter is essential for repression in non–male germline cells. Furthermore, the introduction of silencer sequences from *LGC1* promoter completely repressed *CaMV* promoter (fig. S2, C and D), hence this silencer can confer in planta transcriptional repression in the context of a surrogate promoter. However, *CaMV* promoter carrying randomly selected human genome sequence in the same location showed normal GUS expression in *Arabidopsis* plants (fig. S2E). These results provide confirmation of an evolutionarily conserved repressor system that is capable of regulating genes containing a *LGC1*-type cis-acting silencer sequence.

**Germline restrictive silencing factor (GRSF) is a 24-kD protein.** We identified the putative repressor, GRSF, by screening a lily petal cDNA

expression library with double-stranded 43-bp radiolabeled silencer oligonucleotide, using in vitro binding conditions optimized by electrophoretic mobility shift assay (EMSA) with lily petal nuclear extracts (fig. S3). Screening of nearly 800,000 clones from an unamplified cDNA library led to the selection of four positive clones, the sequencing of which revealed that all four represented the same cDNA of varying lengths. One of these clones contained an 840-bp insert, and we used this sequence to obtain the full-length clone by 5′-RACE (rapid amplification of cDNA ends) (GenBank accession number DQ507850). The open reading frame (ORF) of GRSF cDNA predicts a protein comprising 207 amino acids with a molecular mass of about 24,000 daltons.

A BLAST search of deduced amino acid sequences revealed that the C-terminal portion of GRSF exhibits high similarity to nucleolins such as maize nucleic acid–binding protein (NBP) (8), *Arabidopsis* nucleolin (9), and the potato single-stranded DNA-binding repressor SEBF (10). However, the N-terminal region of GRSF shows arginine/serine-rich motifs that are conserved in SON repressor proteins (Fig. 3A and fig. S4). SON and its isoforms are negative regulatory element–binding proteins that have so far been identified only in humans and other mammalian systems (11). An AT-hook (12) domain, 5-hydroxytryptamine 5B receptor (13), and histone H5 signatures (14) were also detected in the GRSF sequence (Fig. 3A). Proteins containing AT-hook domains bind minor grooves of A/T-rich sequences and are considered to coregulate transcription by modifying the architecture of DNA by recruiting proteins involved in chromatin remodeling and condensation, thus modifying the architecture of the bound DNA (12). In addition, GRSF contains RNA recognition motifs that might mediate nuclear RNA processing activity in addition to its probable transcriptional regulatory role. GRSF contains a domain with the potential to adopt a coiled-coil structure, which has been reported in several transcription factors (15). Comparison of the biochemical properties of GRSF to those of nucleolins, ribonucleoproteins, and known plant repressors shows that GRSF has the lowest molecular weight but the highest arginine content (16.4%) and a calculated isoelectric point of 10.40. All these observations point toward GRSF being a novel eukaryotic DNA-binding repressor protein.

**GRSF is localized in nuclei of non–male gamete lineage cells.** GRSF transcripts are present at high levels in leaf and petal tissues but at moderate levels in pollen and ovary tissues. No signal was detectable in generative cells (Fig. 3B). The positive signal from pollen RNA and the absence of signal from isolated generative cells show that GRSF transcripts are present in the vegetative cells of pollen. Low signal from total pollen RNA is not unexpected, as GRSF is expressed in one cell of pollen only. Although a lower level of GRSF expression in ovary tissues is
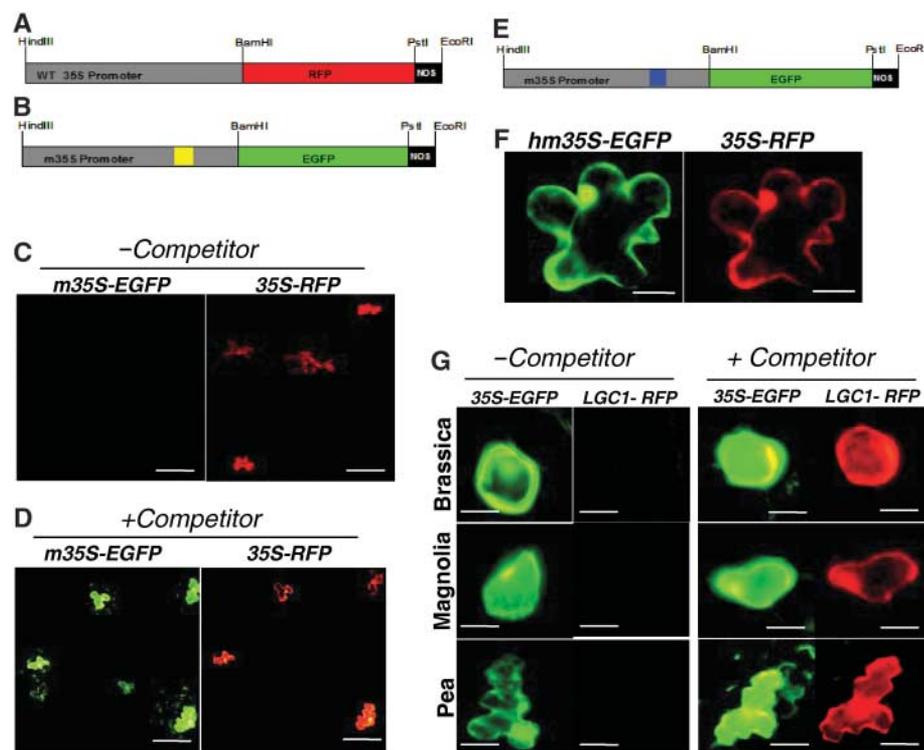


**Fig. 2.** *CaMV35S* promoter becomes inactive when modified by insertion of silencer region from *LGC1* promoter. This silencing can be reversed by cotransformation with competitor. (**A**) Schematic representation of the wild-type (WT) *CaMV35S-RFP*. Digestion enzymes are noted. (**B**) Schematic representation of the modified *CaMV35S-EGFP* harboring the *LGC1* silencer region. Yellow box: AGATTTATCAGTGGCTGAATTTGGGTGCTGTAGAGACAGAATT. (**C**) Cotransformation of lily petal cells with modified *CaMV35S-EGFP* and wild-type *CaMV35S-RFP* shows expression of RFP only. Scale bar, 200 μm. (**D**) However, cotransformation of modified *CaMV35S-EGFP* with excess competitor (100:1) resulted in its reactivation, resulting in expression of GFP. The same cells show RFP expression due to activity of wild-type *CaMV35S-RFP*. Scale bar, 200 μm. (**E**) Schematic representation of insertion of random human sequence in *CaMV35S-EGFP*. Blue box: TCTCTTACACAGGCAATGATGACATCATCATGACCTCTAAAGA. (**F**) Insertion of random human sequence in *CaMV35S-EGFP* had no effect on expression of GFP; *CaMV35S-RFP* was used as a control. Scale bar, 25 μm. (**G**) *LGC1-RFP* normally inactive becomes activated by excess of competitor (100:1), showing expression of the RFP in petal tissues of diverse plants tested. *CaMV35S-EGFP*, used as an internal control, shows activity irrespective of the presence or absence of competitor. Scale bar: *Brassica*, 10 μm; *Magnolia* and pea, 25 μm.

intriguing, transcript levels are not always tightly linked to cellular levels of protein products (*16*). The deduced amino acid sequence of GRSF contains a bipartite nuclear targeting sequence and a putative arginine-rich nuclear localization signal. Immunolocalization experiments using antibodies to GRSF showed signal in the nucleus of lily uninucleate microspores, in the vegetative cell nucleus of the bicellular stage of pollen development, and in anther wall cells (Fig. 3C); however, no signal was detectable in the generative cell nucleus.

To determine whether GRSF protein targeted chromatin *LGC1* silencer elements in vivo, we performed chromatin immunoprecipitation (ChIP) with antibodies to GRSF. The chromatin fragments that coimmunoprecipitate with GRSF were analyzed by real-time quantitative polymerase chain reaction (PCR) using primers specific for promoter and ORF sequences of *LGC1*, generative cell–specific histones, and the pollen vegetative cell–specific pectate lyase gene. Our results confirmed that GRSF occupies a specific domain in the promoter region of *LGC1* (Fig. 3D). We also analyzed the presence of GRSF on the promoter of the generative cell–specific histone gene *gcH3* (*17*). We observed that *gcH3* also shows specific immunoprecipitation with antibodies to GRSF (Fig. 3D). In addition, our analysis of the pollen-expressed pectate lyase gene as a control showed no association of GRSF with this vegetative cell–expressed gene.

These ChIP results provide direct evidence that promoters of *LGC1* and other generative cell–specific genes are likely targets for GRSF-mediated transcriptional repression. Our results thus show a direct correlation between the recruitment of GRSF to the upstream sequences of specific genes and their male gametic cell–specific expression.

**Core silencer sequences are conserved in various male germline–specific genes.** We used radio-labeled 43-bp double-stranded silencer sequence oligonucleotide in an EMSA test of its binding to recombinant GRSF (fig. S5). Further EMSA experiments using mutated oligonucleotides containing blocks of 10-bp mutations showed that mutant 3 corresponded to nucleotide sequence critical for the binding of GRSF (Fig. 4A). A series of oligonucleotides that carried blocks of 4-bp mutations were then used as cold competitors with radiolabeled wild-type 43-bp oligonucleotide. All of the mutants except mutants 7 and 8 nearly abolished the binding of labeled wild-type oligonucleotide (Fig. 4B). The partial inhibition of binding by mutants 7 and 8 suggests that the sequences (GGCT and GAAT) altered in these mutants form a component of the optimal binding site for GRSF. The 8-bp sequence motif represented by both mutants 7 and 8 is also contained within the 11-bp repressor-binding motif defined by mutant 3 (Fig. 4A), thus identifying it as the core silencer domain recognized by GRSF.

Our search for similar cis-acting silencers in other male germline–specific genes showed a similar conserved motif with four invariant bases (Fig. 4C). These genes include male gamete–specific histone *gcH3* of lily (*4,17*), male gamete–specific histone H3 variant of *Arabidopsis* (*18*), and three additional *Arabidopsis* genes that include *DUO1* (*5*) and *At5g49150* (*6*). It is noteworthy that out of 15 histone H3 genes in the *Arabidopsis* genome (*18*), only the male germline–specific H3 contains the core GRSF-binding domain. The recruitment of GRSF to the silencer motif of lily *LGC1* and *gcH3*, as shown by ChIP assay and the presence of a similar silencer motif in three *Arabidopsis* genes, suggests that they could be direct target genes of GRSF or a similar functionally conserved repressor. Our database search for a GRSF-type repressor indeed showed the presence of similar

expressed sequence tags in *Medicago*, maize, rice, *Arabidopsis*, wheat, and *Hordeum* (fig. S6).

**Conclusions.** Conservation of the repressor-binding site and its associated repressor in phylogenetically distant plants suggests that specific repressor binding element–mediated silencing may be a general mechanism for regulating the expression of male germline–specific genes. Our data show that flowering plant male germline–specific genes are maintained in a repressed state in non–male germline cells via negative transcriptional regulation mediated by GRSF or its functional orthologs that are ubiquitously present in nonmale gametic cells. The presence of GRSF in uninucleate microspores but its absence in one of the daughter cells (the generative cell), with corresponding activation of the male germline–specific transcriptional program, suggests that release from GRSF-imposed repression is a determining event in sperm cell development of flowering plants. Through its regulation of germline-specific genes such as *DUO1* that are essential for gamete development, GRSF may function as a key element of a network of regulatory controls of male gamete development. The presence of a GRSF with conserved binding in the basal angiosperm *Magnolia* suggests that the recruitment of GRSF as a regulatory factor controlling the timing and location of expression of male germline genes might be one of the key processes in the evolution of the reproductive system of flowering plants.

The importance of GRSF in controlling a key developmental event in plant biology is comparable to that of neuron-restrictive silencing factor [NRSF; also known as REST (repressor element–1 silencing transcription factor)] for animal systems. NRSF/REST is an evolutionarily conserved repressor with homologs in various species (*Caenorhabditis elegans*, *Drosophila*, *Xenopus*, mouse, and human)

**Fig. 3.** Identification, cloning, expression, and ChIP analysis of repressor protein with specificity toward the *LGC1* silencer domain. (**A**) Schematic representation of predicted functional domains on the repressor (GRSF). NLS, nuclear localization signal; RRM, RNA binding motifs. The GRSF domain with the potential to adopt a coiled-coil structure is shaded. (**B**) Reverse transcription PCR analysis showing *GRSF* mRNA expression in various lily tissues with the exception of generative cells (GC). (**C**) Nuclear localization of GRSF in the nuclei of uninucleate microspores (N) and the vegetative cell nucleus (VN) of mature bicellular pollen. Anther wall cells also exhibit nuclear localization (N) of GRSF. Scale bars, 100 μm. (**D**) GRSF binds to the silencer region of *LGC1* promoter in vivo. ChIP assay of *LGC1* and generative cell–specific histone *H3* promoter (gcH3) used antibodies to GRSF peptide. The data represent the ratio of the amount of DNA immunoprecipitated using specific antibody to that when antibody to GRSF was omitted, as determined by quantitative real-time PCR. P-lyase, pectate lyase.
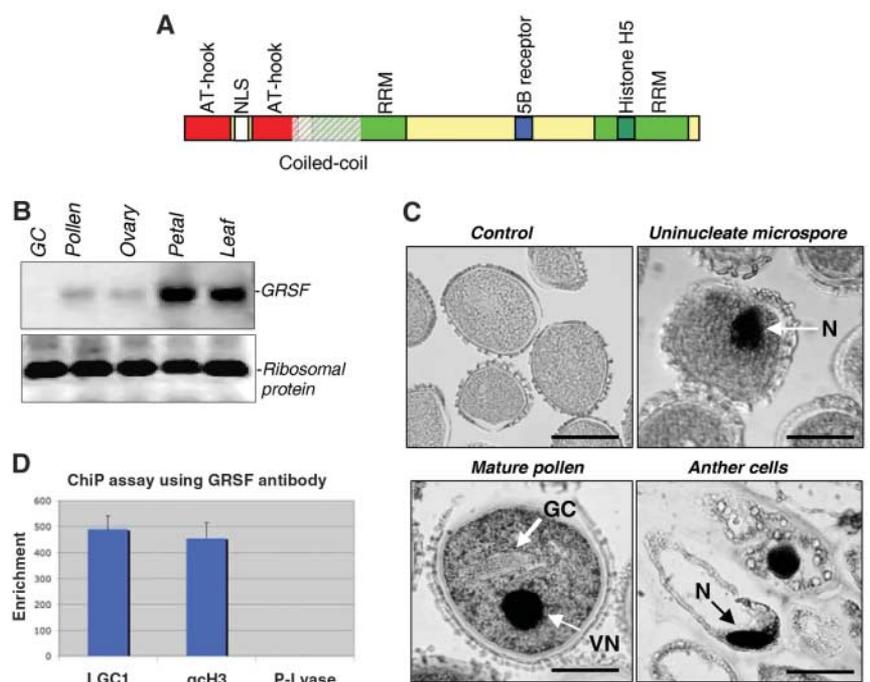
that represses transcription from promoters of numerous neuron-specific genes in neural precursors and non-neuronal cells (*19*) (fig. S7). Silencing of neural-specific genes is mediated via recruitment of the corepressor CoREST, which functions as a molecular beacon for the recruitment of specialized silencing machinery (*19*). The question of whether GRSF-induced silencing of male germline–specific genes in the rest of the plant cells involves associated corepressor(s) and the nature of the silencing machinery required for long-term repression remain exciting areas for further investigation.

**Fig. 4.** Identification of core binding domain of GRSF within the silencer region of *LGC1* promoter and conservation of core silencer domain in male germline genes. (**A**) EMSA using recombinant GRSF shows specific binding to 43-bp oligonucleotide sequence of the *LGC1* promoter (WT). Mutations in the region GGCTGAATTT of the oligonucleotide abolished specific binding (M3); mutations in other regions (M1 and M2) had no effect on binding. Mutated sequences are in red. (**B**) LGC1 oligonucleotides (43 bp) carrying 4-bp mutation blocks (marked in red) used as cold competitors in EMSAs with concentration ratios of 100:1. Mutated oligonucleotides 7 and 8 exhibited the lowest capacity to compete with labeled WT probe. An 8-bp sequence covered by these two oligonucleotides lies within the 10-bp region GGCTGAATTT identified by 10-bp block mutations. (**C**) Conservation of GRSF minimal binding site in the promoter regions of lily and *Arabidopsis* male germline–specific genes. *AT1G19890* encodes *Arabidopsis* male germline–specific



H3 histone, *AT5G49150* encodes *Arabidopsis* male germline–specific unknown gene, and *AT3G60460* encodes *Arabidopsis DUO1* gene expressed in male germline cells. Core binding domain is shaded in yellow, with conserved sequences marked in blue italics.
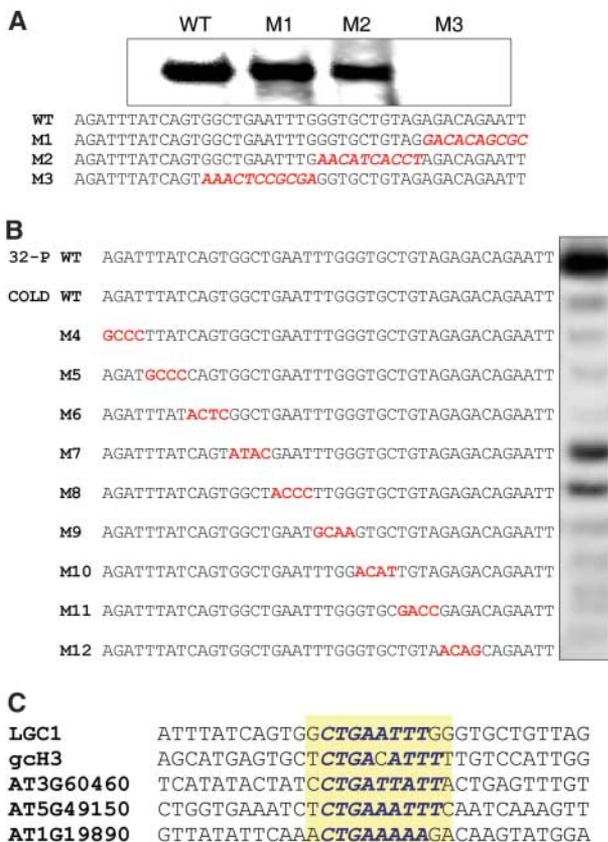
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REPORTS

# Violation of Kirchhoff's Laws for a Coherent *RC* Circuit

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What is the complex impedance of a fully coherent quantum resistance-capacitance (*RC*) circuit at gigahertz frequencies in which a resistor and a capacitor are connected in series? While Kirchhoff's laws predict addition of capacitor and resistor impedances, we report on observation of a different behavior. The resistance, here associated with charge relaxation, differs from the usual transport resistance given by the Landauer formula. In particular, for a single-mode conductor, the charge-relaxation resistance is half the resistance quantum, regardless of the transmission of the mode. The new mesoscopic effect reported here is relevant for the dynamical regime of all quantum devices.

For a classical circuit, Kirchhoff's laws prescribe the addition of resistances in series. Its failure has been a central issue in developing our understanding of electronic transport in mesoscopic conductors. Indeed, coherent multiple electronic reflections between scatterers in the conductor were found to make the conductance nonlocal (*1*, *2*). A new compo-sition law of individual scatterer contribution to resistance was found that led to the solution of the problem of electron localization (*3*) and, later, to formulation of the electronic conduction in terms of scattering of electronic waves (*4*). Nonadditivity of series resistances, or of parallel conductances, nonlocal effects, and negative four-point resistances (*5*) have been observed in a series of transport experiments at low temperature, where phase coherence extends over the mesoscopic scale (*6*, *7*). It is generally accepted that the conductance of a phase-coherent quantum conductor is given by the Landauer formula and its generalization to multilead conductors (*8*), which relate the conductance to the transmission of electronic waves by the conductance quantum $e^2/h$. But, how far is this description robust at finite frequency, where conductance combines with nondissipative circuit elements such as capacitors or inductors? Are there significant