ilar to that seen in ChAT knockout mice (*17*). This may result from perturbed neuronal activity. The most unique aspect of the *reeler* phenotype is the failure to achieve input elimination that is normally completed by the end of the second postnatal week (*10*). NCAM deficiency (*18*) or the overexpression of trophic factors (*10*) caused a delay of synaptic editing due to continued remodeling and axonal sprouting. In *reeler*, however, sprouting was not observed, and multiple innervation was seen even in adult animals. Exogenous Reln promoted axonal withdrawal, strongly suggesting that Reln regulates synapse elimination. To our knowledge, Reln is the first identified molecule that is required for this process in vivo. Our pharmacological studies, demonstrating the mechanism of Reln activity, are consistent with previous studies that invoked the action of a serine protease in the removal of multiple terminals from the NMJ (*19*, *20*). Synapse elimination is a competitive phenomenon affected by electrical activity. We hypothesize that this activity may modulate Reln function, for example, by affecting its local concentrations in the synaptic cleft.

The absence of Reln in humans results in lissencephaly with cerebellar hypoplasia, a disease characterized by ataxia and, notably, abnormal neuromuscular connectivity (*21*). Genes disrupted in congenital forms of muscle dystrophy were recently implicated in neuronal migration (*22*). The requirement for Reln in both brain and muscle development further demonstrates that the extracellular environments of these tissues share essential molecules. Our findings suggest that Reln may affect synaptic maturation in the brain. Distinct alterations in cerebellar (*23*), hippocampal (*24*), and retinal circuitry (*25*) have been reported in *reeler* mice, and the number of dendritic spines is reduced in *reeler* heterozygous mice (*26*). Reln also affects long-term potentiation in the hippocampus (*27*). We speculate that Reln deficiency could lead to abnormal neuronal connectivity and that this could underlie the insurgence of cognitive disorders associated with Reln abnormalities, such as schizophrenia (*28*) and autism (*29*).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/301/5633/649/DC1
Materials and Methods
Figs. S1 to S4
References

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Genome-Wide Insertional Mutagenesis of *Arabidopsis thaliana*

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Over 225,000 independent *Agrobacterium* transferred DNA (T-DNA) insertion events in the genome of the reference plant *Arabidopsis thaliana* have been created that represent near saturation of the gene space. The precise locations were determined for more than 88,000 T-DNA insertions, which resulted in the identification of mutations in more than 21,700 of the ~29,454 predicted *Arabidopsis* genes. Genome-wide analysis of the distribution of integration events revealed the existence of a large integration site bias at both the chromosome and gene levels. Insertion mutations were identified in genes that are regulated in response to the plant hormone ethylene.

One of the most significant findings revealed through analysis of genomes of multicellular organisms is the large number of genes for which no function is known or can be predicted (*1*). An essential tool for the functional analysis of these completely sequenced genomes is the ability to create loss-of-function mutations for all of the genes. Thus far, the creation of gene-indexed loss-of-function mutations on a whole-genome scale has been reported only for the unicellular budding yeast *Saccharomyces cerevisiae* (*2–4*). Although targeted gene replacement via homologous recombination is extremely facile in yeast, its efficiency in most multicellular eukaryotes does not yet allow for the creation of a set of genome-wide gene disruptions (*5*, *6*). Gene silencing has recently been used to study the role of ~86% of the predicted genes of the *Caenorhabditis elegans* genome in several developmental processes (*7*, *8*). The RNA interference (RNAi) method has, however, several drawbacks, including the lack of stable heritability of a phenotype, variable levels of residual gene activity (*9–11*), and the inability to simultaneously silence several unrelated genes (*12*).

somes are known to have lower gene density and a higher concentration of transcriptionally silent transposons and pseudogenes (*1*, *17*).

Next, we examined the preference for T-DNA insertion events within particular genetic elements, including 5′ and 3′ untranslated regions (UTRs), coding exons, introns, and predicted promoter regions (*13*). The coordinates for each of these elements were deduced either from full-length cDNA sequences, which are available for 11,930 genes (table S2), or from gene predictions from the latest release of the *Arabidopsis* genome annotation (table S1). No significant differences were observed between the frequencies of insertion events in 5′UTRs versus 3′UTRs, nor were there differences between coding exons versus introns [Table 1, fig. S2, and (*13*)]. However, a significant bias was seen against integration events in introns and coding exons in favor of 5′UTR, 3′UTRs, and promoters. Moreover, when all intergenic regions were compared with all genes, we detected a small bias toward T-DNA insertions in the intergenic regions. Although there were no effects of G + C content on T-DNA integration sites observed at the genome scale (*13*), we found a positive correlation between the G + C content and the number of insertions in promoters, 5′UTRs, exons, and intergenic regions. Similarly, we detected a negative correlation and no correlation between the G + C content and insertion frequency in introns and 3′UTRs, respectively (fig. S2).

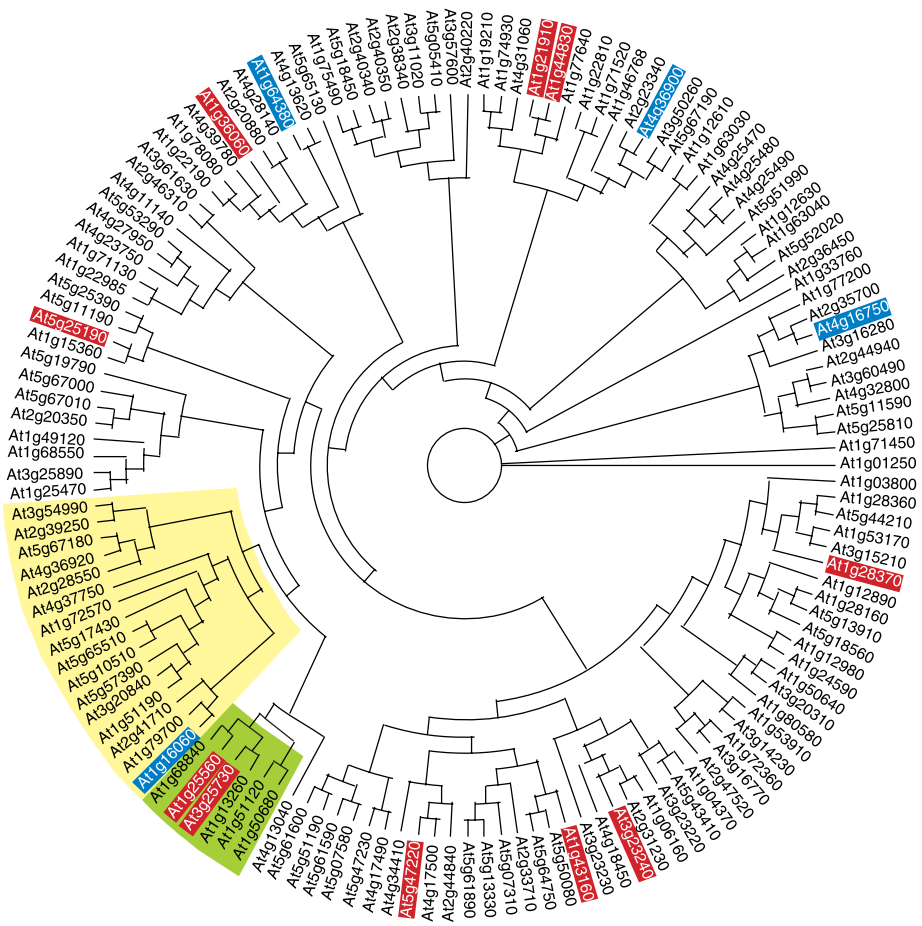

Fig. 2. Functional analysis of the AP2/EREBP multigenic family. Neighbor-joining tree of AP2 domain–containing proteins in *Arabidopsis* was constructed with ClustalWPPC and PAUP3.1.1 software (see supplementary data). The non-EREBP AP2-containing proteins are on the same branch of the tree and are highlighted in yellow. Six EREBP-like genes closely related to the *AINTEGUMENTA* family (*EDFs*) are highlighted in green. AP2 domain–encoding genes that were induced or repressed by ethylene by a multiple of at least two are highlighted in red and blue, respectively. Insertions in promoters or transcribed regions were found for 69 of the 141 AP2 domain–encoding genes and are marked with asterisks.

Table 1. Distribution of T-DNA insertions in genes and intergenic regions.

	Chr. 1	Chr. 2	Chr. 3	Chr. 4	Chr. 5	Total
Promoter	5,488	3,376	4,452	3,076	4,900	21,292
5′UTR	1,243	737	951	680	1,099	4,710
Coding exon	5,089	2,960	3,988	2,871	4,440	19,348
Intron	2,663	1,507	1,840	1,681	2,284	9,975
3′UTR	1,621	914	1,263	966	1,535	6,299
Intergenic regions	6,861	4,323	5,180	3,813	6,321	26,498
Total	22,965	13,817	17,674	13,087	20,579	88,122

Although the precise mechanism of T-DNA integration in the host genome is not fully understood, a variety of host proteins appear to play important roles not only in T-DNA transport but also in integration processes (*18*). For example, the plant VIP2 protein, which is thought to interact with the transcriptional machinery, directly interacts with *Agrobacterium* VirE2 (a bacterial protein associated with the T-strand) (*19*). It is conceivable that the bias toward promoters and UTRs is the result of preferential interaction of the Vir proteins with host proteins involved in initiation or termination of transcription.

As recently reported for HIV integration into the human genome, the process of DNA integration can be significantly affected by gene activity (*20*). Thus, another plausible model for integration site preference is that uncoiling of the DNA helix during transcription initiation and termination at 5′ and 3′UTRs may allow greater accessibility to the T-DNA integration machinery (*20*). To test this possibility, we assessed genome-wide gene expression levels using unique expressed sequence tags (ESTs) present in GenBank for each *Arabidopsis* gene, as well as microarray analysis to examine the expression levels for ~22,000 genes in plants grown under a variety of different conditions (table S3). We observed no significant correlation between the level of gene expression and the frequency of T-DNA integration. Caveats of this conclusion are that the exact cell-type infected by *Agrobacterium* is not known and that mixed-stage flowers may not be adequately representative of expression in the highly specialized female gametophyte cells that are the most likely target for transformation (*21*, *22*).

To test the utility of the sequence-indexed *Arabidopsis* insertion mutant collection for genome-wide functional analysis, we targeted genes in the response pathway of the plant hormone ethylene (*23*). This simple hydrocarbon is an essential regulator of plant disease resistance, fruit ripening, and a variety of other important developmental processes in plants. The transcriptional activation of genes in response to ethylene depends on the plant-specific EIN3 and EIN3-like (EIL) family of DNA binding proteins, and these

are among the most downstream signaling components in the ethylene pathway (*23*). Thus far, the only described direct target of this family of transcription factors is *ERF1* (*ETHYLENE RESPONSE FACTOR1*) (*24*), a member of a large family of AP2-like DNA binding transcription factors known as EREBPs. To identify new genes involved in responses to this important plant-growth regulator, we used Affymetrix gene expression arrays to examine the RNA levels of more than 22,000 genes in response to ethylene (*13*). We identified 628 genes whose levels of expression were significantly altered by treatment with exogenous ethylene; 244 genes were induced and 384 genes were repressed by hormone treatment (table S4). The distribution according to ontology of these genes indicated that ethylene affected genes involved in many types of biological processes, from metabolism to signal transduction (*16*). In total, by searching our sequence-indexed T-DNA insertion-site database (*25*), T-DNA insertion mutations for 179 inducible and 287 repressible genes were identified (i.e., for 74.2% of all ethylene-regulated genes) (*26*). This percentage is in agreement with the total proportion of genes disrupted in this collection (74%). Of particular interest, in addition to *ERF1*, we found that the expression levels of 14 of 141 AP2 domain–containing genes were affected by ethylene treatment of etiolated seedlings (Fig. 2). In particular, four of six genes that encode proteins with two plant-specific DNA binding domains, AP2 and B3, were found to be ethylene-inducible [(*16*), Fig. 3B]; these genes were named *ETHYLENE RESPONSE DNA BINDING FACTORS1* to *4*.

By searching the sequence-indexed insertion mutant database and using gene-specific PCR primers with a multidimensional DNA pooling approach, we were able to identify insertion mutant plants for each of the *EDF* family members (*13*). Although no detectable alterations in morphology were observed in the ethylene responses in any of the single mutants (Fig. 3, A and B), we found significant ethylene insensitivity in multiple-mutant plants (Fig. 3, C and D). These findings reveal an important role for the *EDF1* to *4* genes in the response to ethylene. Moreover, the lack of observed phenotypes in the individual *edf* mutants implies a significant degree of functional overlap among the *EDF* gene family members. The residual ethylene sensitivity observed in the quadruple-mutant plants is consistent with the fact that the *EDF* genes represent only one branch of the ethylene response.

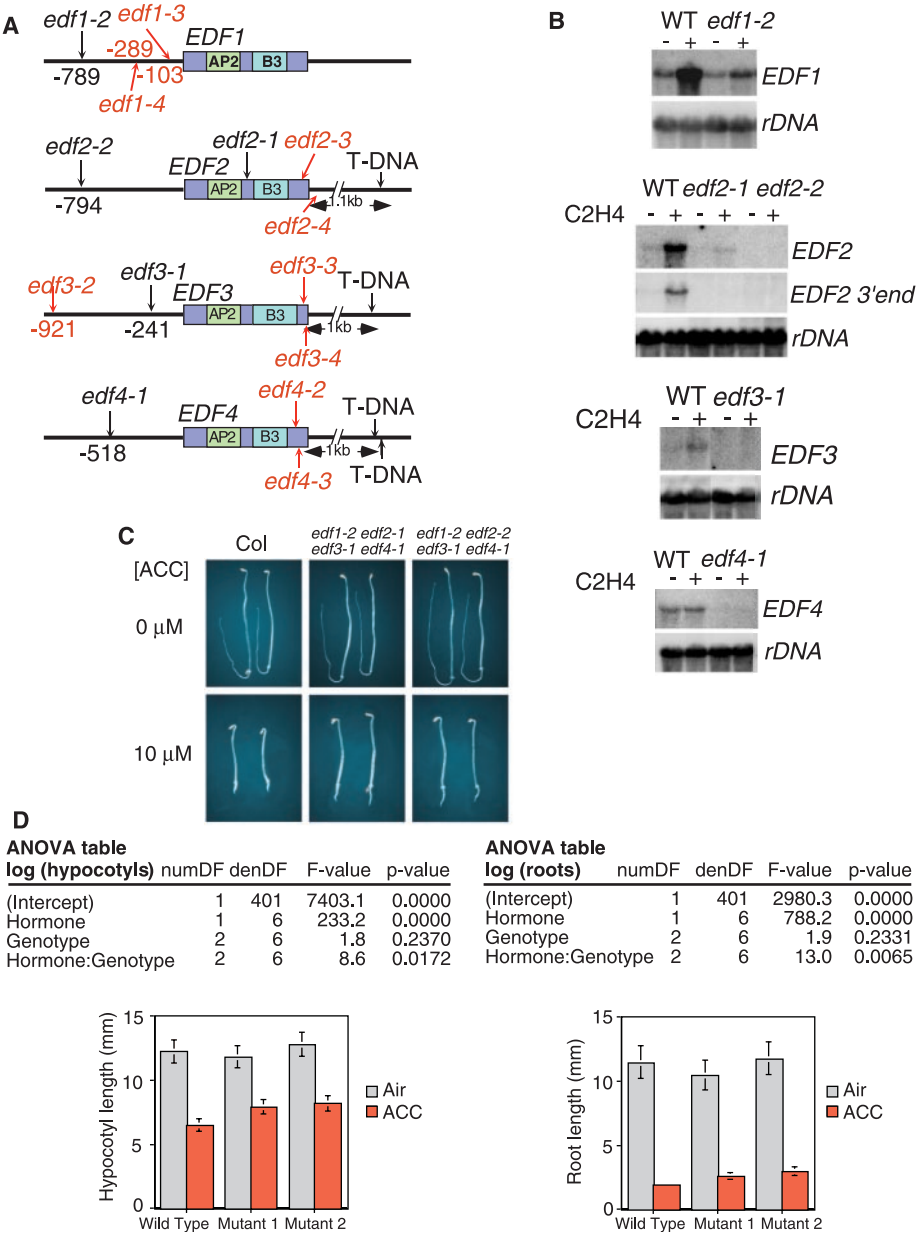

Fig. 3. *EDF* knockouts. (**A**) Schematic representation of the four *EDF* family members with the respective positions of the T-DNA insertions. AP2 and B3 domains are highlighted. The coordinates of the T-DNA insertions in the promoter regions are indicated with respect to the translation start site. Insertions marked in black and red were identified by PCR screening and database search, respectively. (**B**) Expression levels of the *EDF* genes in wild-type plants and the T-DNA mutants with or without 10 ppm ethylene. Total RNA was loaded at 30 µg per lane. (**C**) Ethylene-insensitive phenotypes of two different quadruple-mutant combinations. Wild-type and quadruple mutants were cold-treated and germinated as described in (*27*) for 5 days. (**D**) ANOVA tables for the logarithm of root and hypocotyl lengths. The hormone:genotype term indicates that both quadruple mutants respond to the hormone treatment significantly differently from the wild type. Error bars indicate 95% confidence interval.

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Supporting Online Material
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Screening for Nitric Oxide–Dependent Protein-Protein Interactions

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Because nitric oxide (NO) may be a ubiquitous regulator of cellular signaling, we have modified the yeast two-hybrid system to explore the possibility of NO-dependent protein-protein interactions. We screened for binding partners of procaspase-3, a protein implicated in apoptotic signaling pathways, and identified multiple NO-dependent interactions. Two such interactions, with acid sphingomyelinase and NO synthase, were shown to occur in mammalian cells dependent on endogenous NO. Nitrosylation may thus provide a broad-based mechanism for regulating interactions between proteins. If so, systematic proteomic analyses in which redox state and NO bioavailability are carefully controlled will reveal a large array of novel interactions.

Signal transduction is often coordinated within the confines of multiprotein complexes (*1*). Constitutive interactions among proteins subserve the initiation of signaling, and the emergence of higher order complexes that are required for signal processing and propagation relies on inducible, regulated protein-protein interactions (*1*, *2*). These dynamic interactions form the basis of complex regulatory circuits whose composition determines biological function. Identifying the makeup of these circuits is a major challenge, however, because they involve multiple low-affinity interactions that are controlled by dynamic posttranslational protein modifications in response to multiple stimuli (*2*).

Nitric oxide (NO) is a ubiquitous signaling molecule that operates through posttranslational modification of proteins (nitrosylation) (*3*). Specifically, NO targets cysteine thiol and transition metal centers to regulate a broad functional spectrum of substrates, including all major classes of signaling proteins. NO synthases (NOS) are localized within multiprotein signaling complexes (*3*, *4*), but whether NO can directly affect protein-protein interactions subserving signal transduction has not been considered, and prior biochemical and genetic analyses of protein-protein interactions have been carried out in the absence of NO.

To assess the possibility of NO-dependent regulation of protein-protein interactions in cells, we developed a modified yeast two-hybrid screening methodology (*5*). We first deleted the yeast flavohemoglobin (*6*), which consumes NO very efficiently and thus obfuscates NO signaling. We then established three complementary methods to identify NO-dependent protein-protein interactions. NO was delivered from a long-lived donor (Z)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl) amino]diazen-1-ium-1,2-diolate (DETA-NO; half-life ~18 hours in our assay) dispersed in solid agar, soft agar, or liquid medium, thus covering a range of nitrosylating conditions. We further established a concentration range of DETA-NO over which physiological amounts of NO could be generated in our assays without impairing yeast growth (fig. S1) (100 μM DETA-NO produced steady-state concentrations of ~300 nM NO in yeast culture medium, as determined with an NO electrode).

A well-characterized example of functional regulation by *S*-nitrosylation is the control of caspase-3–dependent death signaling. *S*-nitrosylation inhibits and denitrosylation facilitates the sequential activation of caspases within macromolecular complexes (*7*–*9*). However, the molecular mechanisms of action that enable *S*-nitrosylation to regulate signal transduction through these complexes remain poorly understood. We therefore conducted a two-hybrid screen of a cDNA library derived from cytokine-activated murine macrophages [which are frequently used to study the involvement of NO in apoptosis (*10*)] in the presence of NO. We initially screened ~4 million transformants for proteins that interact with procaspase-3 (bait). Thirty-five clones were isolated, from which prey plasmids retransfected into the bait strain caused at least threefold increases in growth in the presence but not the absence of NO. No growth was seen in yeast transformed with either bait or prey vector alone. Seventeen of these 35 clones showed NO-dependent growth a third time; 2 of 17 clones also showed at least threefold activation of *lacZ* transcription, an independent reporter of protein-protein interactions, in the presence versus absence of NO ($P < 0.001$). Thus, these clones both activated *lacZ* transcription and conferred histidine prototrophy in an NO-dependent manner. One of these clones contained a partial sequence (amino acids 158 to 927) of the apoptosis-related enzyme, acid sphingomyelinase (ASM) (Fig. 1A). These data establish the principle of NO-inducible protein-protein interactions.

To enable rapid large-scale screening, we developed an alternative method of genomic two-hybrid analysis in which *Escherichia coli* were transformed with plasmids pooled from yeast transformants that were previously grown for several days in histidine-deficient medium, supplemented continuously with NO (*5*). Growth in liquid medium circumvented the requirement for large numbers of plates, and thus for upfront processing of individual clones, and provided better control over NO delivery. The prey plasmids were isolated and retransfected into caspase-3 bait strains, whose NO-dependent growth was then assessed individually. About 1800 *E. coli* colonies were derived from a screen of ~4 million transformants; 499 of these contained prey plasmids, of which 50 appeared at least twice as determined by Hae III diges-

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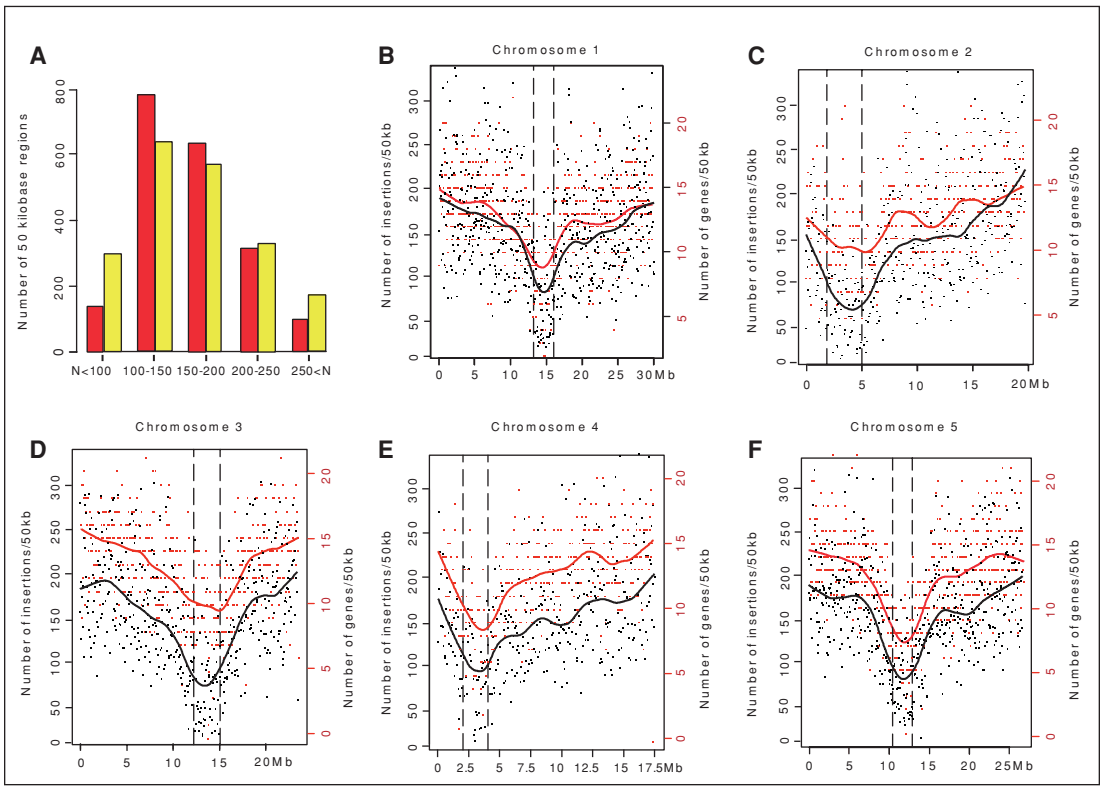
ERRATUM

post date 26 September 2003



REPORTS: "Genome-wide insertional mutagenesis of *Arabidopsis thaliana*" by J. M. Alonso *et al.* (1 Aug. 2003, p. 653). There were errors in two of the figures. In Fig. 1A (above), there was a bar missing from the graph. In Fig. 2 (on the following page), genes in which insertions in promoters or transcribed regions were found should have been marked with asterisks. The corrected figures are shown here.

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post date 26 September 2003, continued