SGR2, a Phospholipase-Like Protein, and ZIG/SGR4, a SNARE, Are Involved in the Shoot Gravitropism of Arabidopsis

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In higher plants, the shoot and the root generally show negative and positive gravitropism, respectively. To elucidate the molecular mechanisms involved in gravitropism, we have isolated many shoot gravitropism mutants in Arabidopsis. The sgr2 and zig/sgr4 mutants exhibited abnormal gravitropism in both inflorescence stems and hypocotyls. These genes probably are involved in the early step(s) of the gravitropic response. The sgr2 mutants also had misshapen seed and seedlings, whereas the stem of the zig/sgr4 mutants elongated in a zigzag fashion. The SGR2 gene encodes a novel protein that may be part of a gene family represented by bovine phosphatidic acid–preferring phospholipase A1 containing a putative transmembrane domain. This gene family has been reported only in eukaryotes. The Zig gene was found to encode AtVTI11, a protein that is homologous with yeast VTI1 and is involved in vesicle transport. Our observations suggest that the two genes may be involved in a vacuolar membrane system that affects shoot gravitropism.

INTRODUCTION

Plants settle in their place of germination through their lifetime and cannot escape the various environmental stimuli to which they are exposed. Consequently, plants have evolved many mechanisms by which they can sense and adapt themselves to various environmental changes. Gravitropism is one of those important environmental responses, particularly for land plants. This is the response that the plant makes when it is laid flat on the ground, namely, the shoot curves up (negative gravitropism) and the root curves down (positive gravitropism). This response is necessary to position the plant so that its leaves face the source of light energy and its roots can take up water and various nutrients.

A number of physiological and cytological studies using many different species of plants have demonstrated that amyloplasts that accumulate starch are involved in gravity perception as statoliths and that auxin is involved in the asymmetric growth between the upper and lower tissue of the responding organ that results in the gravitropic curvature (Sack, 1991; Kaufman et al., 1995). These studies also have suggested that various signal molecules, such as Ca2+, calmodulin, and inositol 1,4,5-triphosphate, and pH change are involved in the signal transduction that generates the gravitropic response (Belyavskaya, 1996; Sinclair and Trewavas, 1997; Perera et al., 1999; Scott and Allen, 1999; Fasano et al., 2001).

To elucidate the molecular mechanism of the gravitropic response in higher plants, many mutants with aberrant root or shoot gravitropism have been isolated from Arabidopsis (Firn et al., 2000; Tasaka et al., 2001). With respect to root or hypocotyl gravitropism, most of the mutants that are abnormal in this function also respond abnormally to auxin treatment. This suggests that a number of auxin-related genes are involved in this response. These include the AUX1 (AUXIN RESISTANT 1) and EIR1/AGR1/PIN2 (ETHYLENE INSENSITIVE ROOT 1/AGRAVITROPIC 1/PIN-FORMED 2) genes, which appear to be involved in auxin transport (Bennett et al., 1996; Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsumo et al., 1998), as well as the AXR1 (AUXIN RESISTANT 1) gene, which encodes a protein similar to the ubiquitin-activating enzyme E1 (Leyser et al., 1993). Also involved may be the MSG1/NPH4 (MASSUGU 1/NONPHOTOTROPIC HYPOCOTYL 4; ARF7) gene, which is a member of the ARF gene family that encodes a putative transcription factor that binds to a specific sequence in the promoter of many auxin-regulated genes (Liscum and Briggs, 1996; Watahiki and Yamamoto, 1997; Harper et al., 2000). Other genes that may belong to the Aux/IAA gene family include AXR2 (IAA7), AXR3 (IAA17), SHY2 (SUPPRESSOR OF HY2; IAA3), MSG2 (IAA19), and SLR (SOLITARY ROOT; IAA14). The expression of these genes is regulated by auxin (Wilson et al., 1990; Timpte et al., 1992; Rouse et al., 1998; Tian and Reed, 1999; Nagpal et al., 2000;
Fukaki et al., 2002). These results indicate that a signaling pathway(s) dependent on auxin is crucial for gravitropism, probably for the differential growth involved in this response.

Another mutant that shows a reduced gravitropic response in both the root and the hypocotyl is the rhg (root and hypocotyl gravitropism) mutant, which is allelic to arg1 (altered response to gravity) (Fukaki et al., 1997). The ARG1 gene encodes a novel protein with a J domain that is conserved in the DnaJ/Hsp40 gene family and with a putative coiled-coil domain that could interact with microtubules or microfilaments (Sedbrook et al., 1999).

With respect to shoot gravitropism, when Arabidopsis inflorescence stems are placed horizontally, they bend upward, and the curvature reaches 90° after ~90 min (Fukaki et al., 1996a). We have isolated a number of sgr (shoot gravitropism) mutants that are abnormal in this function and have found that at least seven loci are involved (Fukaki et al., 1996b, 1998; Yamauchi et al., 1997). It is believed that the transport and distribution of auxin are involved in both gravitropism and phototropism (Kaufman et al., 1995). Because all of the sgr mutants can perform the phototropic response (Fukaki et al., 1996b; Yamauchi et al., 1997), it is believed that the transport and distribution of auxin probably is normal. Thus, it is more likely that the sgr mutants are impaired in either their perception of gravity or the signaling pathway(s) involved in the gravitropic response. The sgr1 and sgr7 mutants, which show an agravitropic response in both the hypocotyl and the inflorescence stem, are allelic to the radial pattern mutant of the root, scr (scarecrow) (Scheres et al., 1995; Di Laurenzio et al., 1996), and shr (short-root) (Scheres et al., 1995), respectively.

The sgr2 and zig (zigzag)/sgr4 mutants are abnormal in the gravitropism of their inflorescence stems and hypocotyls (Fukaki et al., 1996b; Yamauchi et al., 1997). As reported here, we have characterized mutants in detail and have cloned the SGR2 and ZIG/SGR4 genes. SGR2 was found to encode a novel protein that is homologous with the phosphatidic acid–preferring phospholipase A1 (PA-PLA1) found in bovine testis (Higgs et al., 1998). In addition, ZIG/SGR4 was found to encode AtVTI11, which was identified by an Arabidopsis expressed sequence tag (EST) database search as being homologous with the yeast v-SNARE VTI1p (Zheng et al., 1999). This finding suggests that membrane traffic is involved in gravitropism.

RESULTS

Gravitropic Responses of sgr2 and zig/sgr4

After being placed horizontally, Arabidopsis wild-type (Columbia [Col]) inflorescence stems bent upward, and the curvature reached 90° in ~90 min (Figure 1A). In contrast, the

![Figure 1. Shoot Gravitropism of sgr2 and zig/sgr4.](image)

(A) to (C) Gravitropic response of inflorescence stems of 5-week-old plants after 90 min of horizontal gravistimulation.

(A) Wild type (Col).

(B) sgr2-1.

(C) zig-1.

(D) to (F) Gravitropism of 3-day-old etiolated seedlings.

(D) Wild type (Col).

(E) sgr2-1.

(F) zig-1.

The arrow indicates the direction of gravity (g).
sgr2-1 and zig-1/sgr4-1 inflorescence stems showed little response (Figures 1B and 1C), as reported previously (Fukaki et al., 1996b; Yamauchi et al., 1997). In addition, etiolated wild-type seedlings grew upward uniformly, whereas those of sgr2-1 and zig-1/sgr4-1 did so in a disorderly fashion (Figures 1D to 1F). The roots in both mutants showed normal positive gravitropism (Fukaki et al., 1996b; Yamauchi et al., 1997). Thus, SGR2 and ZIG/SGR4 are involved specifically in shoot gravitropism. Apart from their abnormal gravitropic response, the mutants also exhibited some morphological anomalies. In the wild-type plant, primary inflorescence stems grew straight up, as did the lateral shoots (Figure 2A). In sgr2-1, however, the inflorescence stems wound slightly at each node and the lateral shoots rolled downward (Figure 2B). In contrast, the inflorescence stems of zig-1/sgr4-1 elongated in a zigzag fashion, although its lateral shoots tended to curl downward, similar to those of sgr2-1 (Figure 2C). Because of this characteristic stem shape, sgr4 was renamed zigzag.

The kinetics of the gravitropic response of the inflorescence stems from the mutant plants were measured (Figure 3). In the wild-type plant, the stem segments began to bend upward within 30 min and the curvature reached 90° in ~90 min. After bending too far, they curved in the opposite direction and finally settled at 90° (Figures 3A and 3B) (Fukaki et al., 1996a). Four sgr2 alleles were newly isolated in addition to the six alleles reported previously (Fukaki et al., 1996b). The gravitropic response of all 10 alleles was measured, which allowed the alleles to be classified into strong (sgr2-1, sgr2-3), middle (sgr2-5, sgr2-6), or weak (sgr2-10) alleles (Figure 3A and data not shown). The strong alleles showed little gravitropic curvature even after 24 hr, whereas the middle alleles showed some slow gravitropic response, but the curvature did not reach 90° even after 24 hr. The kinetics of response in sgr2-10, which has the weakest allele, were comparable to that of the wild type except that its initial response had a lag time and its later response after bending excessively (after 150 min) was slow (Figure 3A, closed squares).

Two additional alleles of zig were isolated, both of which had the characteristic zigzag-shaped inflorescence stem (data not shown). The stem segments of zig-1 and zig-2 showed little and no response to gravity, respectively, even after 24 hr (Figure 3B). zig-3, however, did show a response to gravity, albeit one that was retarded markedly. These plants started to bend upward within 60 min, after which it took 3 hr to reach 90°. After bending excessively, the curvature settled at 90° after 24 hr.

The Seed and Seedling Phenotype of sgr2

Because sgr2 mutations are recessive (Fukaki et al., 1996b), the segregation ratio of F2 plants produced by the crossing of sgr2 and wild-type plants is expected to be 1:3. However, the incidence of sgr2 mutants was slightly lower than this (data not shown). Although all of the wild-type and most of the sgr2 seed were uniform in their oval shape and size (Figure 4A), some sgr2 seed were irregular in shape and shrunken (Figure 4C). The shrunken seed did not germinate, which may be responsible for the slight decrease in the incidence of mutant F2 plants. After germination, although most sgr2 seedlings were normal in shape, some were abnormal (Figures 4B and 4D). For example, some seedlings had three cotyledons (Figure 4D) or only one cotyledon. Some were even more abnormally shaped (data not shown). All of these abnormally shaped seedlings had an active shoot apical meristem, and the gravitropic response of their inflorescence stems was aberrant.

Map-Based Cloning of SGR2

We performed map-based cloning of the SGR2 gene by analyzing ~1700 chromosomes. SGR2 was found to map 0.8 to 1.0 centimorgan south of m254 on chromosome 1 (Figure 5A). Because sgr2-8, sgr2-9, and sgr2-10, which were mutagenized by fast neutron exposure, were expected to have a deletion in the SGR2 locus, they were used for DNA gel blot analysis. The 10-kb fragment that included the T7 end of bacterial artificial chromosome T4D9 (Figure 5A, probe X) was used as a probe. The polymorphism between the wild-type and sgr2-10 genomes was detected after digestion with Xbal (Figure 5B). Thus, SGR2 is located around the probe X fragment. Approximately 25 kb of the genome that includes the region corresponding to probe X was sequenced (Figure 5A, gray lines). sgr2-10 was shown to have a 10-kb deletion in the sequenced locus, and the deleted fragment seemed to have translocated into a locus 92-kb south (data not shown). The sequenced locus was predicted
to contain more than eight putative coding regions. Finally, the mutations of all sgr2 alleles were mapped to one gene located just to the south of the sgr2-10 deletion site (Figure 5A).

The SGR2 gene was composed of 22 exons and encoded a protein with a molecular mass of 106.3 kD (933 amino acids) (Figure 5C). Both sgr2-1 and sgr2-4, the strong alleles, had the same mutation, namely, a G-to-A transition at the boundary between the 20th exon and the intron. In sgr2-2 and sgr2-5, their respective codons (Arg-358 and Trp-189) had been changed into stop codons by the transition of a single nucleotide (Figure 5C). In both sgr2-6 and sgr2-3, there was a missense mutation that caused the amino acid substitutions of G320S and G446A, respectively. Both Gly residues are highly conserved in the SGR2 gene family (Figure 6B). Four alleles (sgr2-7, sgr2-8, sgr2-9, and sgr2-10) had a deletion in this locus, as shown in Figure 5C.

The ~7.3-kb wild-type genome fragment that extends from 1.2 kb upstream of the putative transcription start site to 400 bp downstream of the putative transcription termination site could complement the shoot gravitropism of sgr2-1 (Figure 5D). This finding indicates that the gene is identical to SGR2. RNA gel blot analysis of the wild-type plant was performed using the 700-bp sequence in the 3′ region of SGR2 as a probe. An ~3.0-kb transcript of SGR2 was detected in all organs, even in the root, which does not show an aberrant response to gravity (Figure 5E). SGR2 mRNA was barely detected in siliques by RNA gel blot analysis but could be detected by nonquantitative reverse transcription–polymerase chain reaction (RT-PCR) (data not shown).

SGR2 Encodes a Protein Homologous with PA-PLA1

SGR2 was found to be a novel and unique gene that does not have a homologous gene in the Arabidopsis genome. In tomato (AW035183, AI486639, AW220938, AW219077, and AW930345), soybean (BE058094 and BE058090), cotton (AW725387), rice (C26667, D43182, and C28346), and maize (AW424734, AW313200, and AW324709), EST clones that are homologous with SGR2 have been registered in the database, suggesting that SGR2-like genes are present in other higher plants. SGR2 also was similar in sequence to proteins from several eukaryotes, such as Schizosaccharomyces pombe (genes SPAC20G8.02 and SPCC1020.13), Saccharomyces cerevisiae (gene YOR022c), Neurospora crassa (gene B14D6.220), Caenorhabditis elegans (gene M03A1.6), Drosophila melanogaster (gene CG8552), and mammals. Thus, these genes constitute a small gene family. Some regions of the gene were highly conserved within the family, but others were not. The proteins of two of the members of this family have been characterized, namely, PA-PLA1, whose gene was cloned from bovine testis (Higgs et al., 1998), and p125, which was identified in human (Tani et al., 1999). The latter has been cloned as a Sec23p-interacting protein and is suggested to be involved in vesicle transport from the endoplasmic reticulum to the Golgi apparatus (Figures 6A and 6B).

SGR2 bears the lipase consensus sequence (GXSXG) that is highly conserved in all members of the gene family except PA-PLA1 (SHSLG). In sgr2-3, an amino acid substitution of

**Figure 3.** Time Course of the Gravitropic Response of Inflorescence Stems. (A) The gravitropic response of sgr2 alleles: wild-type (Col; open circles), sgr2-1 (closed circles), sgr2-3 (open squares), sgr2-5 (closed triangles), sgr2-6 (open triangles), and sgr2-10 (closed squares) stem segments. (B) The gravitropic response of zig/sgr4 alleles: wild-type (Col; open circles), zig-1 (open triangles), zig-2 (open squares), wild-type (Wassilewskija; closed circles), and zig-3 (closed triangles) stem segments. Stem segments were gravistimulated by being placed horizontally at 23°C in the dark. The curvature was measured at the times indicated.
Arg for the last Gly in the consensus sequence caused a comparatively profound inability to respond to gravity (Figures 3A and 6B). This finding suggests that the lipase consensus sequence is important in gravitropism. SGR2 also was predicted by the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html; Hofmann and Stoffel, 1993) to bear a transmembrane domain at residues 669 to 689. This region also is conserved among the various members of the PA-PLA1 gene family and shows sequence similarity to one of the putative transmembrane domains of the retinal degeneration B protein. This protein is an integral membrane protein (Vlhtelc et al., 1991, 1993), which suggests that the members of the PA-PLA1 gene family code for membrane proteins. Another feature of SGR2 was a coiled-coil structure predicted at residues 593 to 635 by the COILS program (http://www.ch.embnet.org/software/COILS_form.html; Lupas et al., 1991). This structure was found in PA-PLA1 (residues 580 to 606) and MO3A1.6 (residues 445 to 481) (Higgs et al., 1998). These predicted coiled-coil domains are located at similar positions between the lipase consensus sequence and the putative transmembrane domain in each of these three proteins, although there is no amino acid sequence similarity (Figure 6A).

**Morphological Characterization of zig**

The mutation of ZIG caused pleiotropic effects on the plant’s morphology. The rosette leaves of the zig mutant were small and wrinkled. More remarkably, both the primary and lateral stems elongated in a zigzag fashion, bending at the nodes in the opposite direction to the cauline leaves or buds (Figure 2C). Their stems curved upward in the internodes. According to the metamer concept (Shultz and Haughn, 1991), Arabidopsis plants produce three types of metamer, with type 1, type 2, and type 3 metamers being rosette, coflorescence-bearing with cauline leaves, and flower-bearing without bract, respectively. Each metamer contains several nodes. In the wild-type plant, the angles formed by two adjoining internodes in the second and third metamer always were close to $180^\circ$, whereas in the zig-1 mutant, the angles always were narrower (Table 1). In particular, in the mutant plant, the angles were narrower in the second metamer than in the third metamer, with those in the second metamer on the distal side from the rosette tending to be narrowest of all. Moreover, the length of the internodes in the second metamer was considerably shorter in zig-1 than in the wild-type plant, particularly the proximal internodes (Table 2). There was no significant difference in the internode length in the third metamer.

To elucidate the cause of these morphological anomalies, the stem tissues were observed with an optical microscope. There were no aberrations in the structure of the shoot apical meristem and the location of peripheral primordia (data not shown). Furthermore, the patterning of the whole tissue was basically normal, although the cell shape and size were affected. In the mature zig-1 stem, the epidermal cells were shorter and wider than those in the wild-type stem (Figures 7A and 7C). The size of the pith cells in the mutant stem varied, and their arrangement was disordered (Figures 7B and 7D). Thus, the zigzag-shaped stem of the zig mutant may be caused not by the abnormal positioning of the peripheral primordia but rather by the aberrant elongation, shape, and arrangement of cells. Aberrations in cell shape also were observed in other tissues of the mutant, including the pavement cells in leaves and trichomes and the epidermal cells in the hypocotyl (data not shown).

**Map-Based Cloning of ZIG**

We cloned the ZIG gene relative to its map position (Figure 8). We performed polymerase chain reaction (PCR)-based mapping of recombination breakpoints in 2422 chromosomes from F2 progeny. The position of the ZIG locus on chromosome 5 was narrowed to an $\sim$380-kb region (Figure 8A). Because zig-1 and zig-2 were isolated from fast neutron-mutagenized lines, it was expected that the molecular lesions on the gene in these mutants would be deletions. To
detect the expected deletion, DNA gel blot analyses were performed on zig-1 and zig-2 using Mitsui P1 clones or transformation-competent artificial chromosome (TAC) clones as probes. When the TAC clone K16M23 was used as a probe, banding patterns that were different from those in the wild type were found simultaneously in zig-1 and zig-2 (data not shown). zig-1 has a genomic discontinuity within the MUL8.15 gene, according to the annotation in the Katsura Arabidopsis data opening site (Figure 8B). The connection between the fifth intron of MUL8.15 and the first intron of the putative MPO12.P7 gene was detected in the zig-1 mutants by thermal asymmetric interlaced PCR (Liu et al., 1995). This is consistent with the mapping results, which found no recombinants between MUL8 and MPO12. The in-

Figure 5. Molecular Cloning of the SGR2 Gene

(A) SGR2 was mapped to lie between the m254 and 7G6 markers on chromosome 1. The numbers of recombinants are indicated under these markers (recombinant chromosomes/analyzed chromosomes). Probe X (XbaI–XbaI fragment derived from F11B17) was used as a probe in DNA gel blot analysis.

(B) DNA gel blot analysis of three sgr2 alleles that were generated by radiation mutagenization. These genomic DNAs, digested with XbaI, were loaded and blotted. A polymorphism was detected between sgr2-10 and Col. Probe X DNA fragment was used as probe.

(C) The structure of the SGR2 gene and the mutation sites of the sgr2 alleles examined. Boxes represent exons: closed boxes, translated regions; open boxes, untranslated regions. SGR2 is encoded by an ~5.8-kb genome fragment that contains 22 exons and 21 introns. Each mutation of 10 sgr2 alleles is mapped in the genome region.

(D) Complementation of sgr2 gravitropism by the wild-type SGR2 genomic region.

(E) RNA gel blot analysis of SGR2 mRNA levels in each wild-type organ. Each lane was loaded with 5 μg of total RNA. R, roots of a 7-day-old seedling; H, hypocotyl of an etiolated seedling grown in the dark for 3 days; L, mature rosette leaves; S, inflorescence stems; B, floral buds, including inflorescence meristem; Si, siliques.
Cloning of SGR Genes 39

tervening region seemed to be present elsewhere in the genome, because the presence of the relevant region could be detected by DNA gel blot analysis. With respect to zig-2, an ~20-kb-long region of the chromosome that included the MUL8.15 gene was deleted. In zig-3, a point mutation was found in the fifth exon of MUL8.15. This mutation is expected to result in the amino acid substitution of an Asp residue for the Gly-142 residue in a predicted coiled-coil domain (Figure 8C). The 5-kb wild-type genomic fragment containing MUL8.15 was cloned and introduced into zig-1 for a complementation test. The resulting transgenic plants showed wild-type morphology (Figure 8D), and their straight inflorescence stems responded normally to gravity (Figure 8E). These results indicate that MUL8.15 is the authentic Zig gene.

Surprisingly, this gene had been reported previously by Zheng et al. (1999) as AtVTI1a. It was identified as a homolog of the yeast v-SNARE VT11 by Arabidopsis EST database search. Yeast Vti1p is involved in multiple vesicle transport steps, including transport from the Golgi apparatus to vacuoles via endosomes (Holthuis et al., 1998; von Mollard and Stevens, 1999) and retrograde transport within the Golgi apparatus (Lupashin et al., 1997; von Mollard et al., 1997). Chromosomal rearrangement had occurred in zig-1, and abnormal transcripts were detected by RT-PCR (data not shown). Even if expressed, however, the putative product of the zig-1 gene would be nonfunctional because it lacks the transmembrane domain and part of the coiled-coil region (Figure 8C). No transcripts were detected in zig-2 by RT-PCR (data not shown). Thus, zig-1 and zig-2 can be regarded as null mutants. Because a highly conserved residue in the coiled-coil domain was substituted in zig-3, the mutant protein probably is reduced in its activity. The extent of the molecular lesion on each allele correlates well with the severity of its physiological (Figure 3B) and morphological (data not shown) phenotypes.

DISCUSSION

We have isolated a number of mutants whose shoots show a reduced gravitropic response. The genes involved in some mutants, namely, SGR1/SCR and SGR7/SHR, were cloned previously and appear to encode transcriptional factors that are essential in the formation of endodermis in both the shoot and the root (Di Laurenzio et al., 1996; Helariutta et al., 2000). This suggests that these two genes are not involved directly in the processes mediating the gravitropic response. Here we have characterized two other gravitropic mutants, sgr2 and zig, and cloned the responsible genes. sgr2 and zig both were abnormal in the gravitropic responses.

Figure 6. Structure of the SGR2 Protein and Its Homologs.

(A) Scheme of SGR2 (Arabidopsis), PA-PLA1 (B. taurus), MO3A1.6 (C. elegans), and p125 (H. sapiens). Boxes represent conserved sequences. aa, amino acids.

(B) Multiple sequence alignment of two regions that are conserved between SGR2 and its homologs. The horizontal line shows the lipase consensus sequence (GXSXG). Two boldface letters indicate the residues that are changed in sgr2-3 and sgr2-6.
of their hypocotyls and inflorescence stems. Direct involvement of these genes in the gravitropic response is expected because mutant plants showed a normal radial pattern of the stem tissues. However, both mutants also exhibited morphological anomalies in organs that are not involved directly in the gravitropic response, suggesting that these genes probably play multiple roles in several organs.

SGR2 Is Similar in Sequence to PA-PLA1

The SGR2 exhibited sequence homology with the PA-PLA1 gene that was cloned from bovine testis. Genes that are similar to PA-PLA1 are found widely throughout the eukaryotes and form a gene family (Figure 6A). All members of this family contain a lipase consensus sequence (GXSXG) (Higgs et al., 1998), although PA-PLA1 is the only member to date actually shown to have lipase activity. Another gene that is relatively well characterized is the Sec23p-interacting protein, p125, that was identified in human (Tani et al., 1999). The biological functions of PA-PLA1 and p125 are unclear. Sequence searching predicts that all of the genes in the PA-PLA1 gene family are present in their respective genomes as single genes, at least in most organisms whose genome sequences are complete or nearly complete (such as S. cerevisiae, C. elegans, D. melanogaster, and Arabidopsis), except for S. pombe, which has two homologous genes. SGR2 does not appear to be essential for viability because the sgr2-7 and sgr2-2 mutants still are viable in spite of severe molecular lesions that may result in the production of nonfunctional gene products (Figure 5C). Similarly, the YOR022c gene in S. cerevisiae also seems to be dispensable, because the YOR022c null mutant grew normally in glucose-rich medium (Winzeler et al., 1999). The dispensable nature of these genes might make it difficult to characterize their biological functions.

SGR2, like some other family members, was predicted to have a transmembrane domain (Figure 6A). Moreover, all members of the family have a highly conserved region homologous with a putative transmembrane domain present in the retinal degeneration B protein (Vithelie et al., 1991, 1993). This finding suggests that the family members might encode membrane proteins.

It has been reported that the central serine (SHSLG) of the lipase consensus sequence is the active nucleophile, because the missense mutation of Ser to Ala in PA-PLA1 reduced its lipase activity (Higgs et al., 1998). When we produced recombinant SGR2 protein in Escherichia coli cells, we could not detect PLA1 activity (data not shown). This result might be attributable to aberrant protein folding or protein modification in E. coli cells. sgr2-3, which has little gravitropic response, has an amino acid substitution of Arg for the last Gly in the lipase consensus sequence (Figures 3A and 6B), suggesting that this conserved sequence is crucial for the function of SGR2.

If SGR2 has phospholipase activity, what could be the molecular function of SGR2 in the gravitropic response? Phospholipase A1 cleaves a phospholipid, a constituent of the membrane lipid bilayer, into a fatty acid and a lysophospholipid. It is conceivable that the degradation of specific phospholipids by phospholipases alters membrane composition and that this changes membrane structure, fluidity, or function. Although the sensing or signaling mechanism(s) involved in gravitropism remains obscure, physiological and electron microscopic analyses suggest that some membrane systems, such as endoplasmic reticulum and plasma membrane, may participate (Sack, 1997; Chen et al., 1999; Zheng and Staehelin, 2001). Thus, SGR2 may play a role in gravitropism by regulating such membrane systems. Alternatively, SGR2 may produce messenger molecule(s) by cleaving phospholipids. Plants possess many phospholipase families, and the functions of phospholipases A2, C,

### Table 1. Angles Forming at Nodes between Two Adjoining Internodes

<table>
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<tr>
<th>Metamer</th>
<th>Wild Type (°)</th>
<th>zig (°)</th>
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<tr>
<td><strong>Second metamer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First node</td>
<td>174.0 ± 1.1</td>
<td>165.8 ± 2.6</td>
</tr>
<tr>
<td>Second node</td>
<td>178.3 ± 0.6</td>
<td>158.7 ± 2.7</td>
</tr>
<tr>
<td>Third node</td>
<td>176.8 ± 1.4</td>
<td>156.5 ± 2.1</td>
</tr>
<tr>
<td><strong>Third metamer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First node</td>
<td>177.1 ± 1.0</td>
<td>167.5 ± 1.4</td>
</tr>
<tr>
<td>Second node</td>
<td>178.4 ± 0.6</td>
<td>166.8 ± 1.8</td>
</tr>
<tr>
<td>Third node</td>
<td>177.1 ± 1.2</td>
<td>162.0 ± 1.3</td>
</tr>
</tbody>
</table>

The inflorescence stems were used after growth had been completed.

a $n = 12$.

b $n = 24$.

c The nodes were numbered from the proximal side of the rosette.

d 180° indicates a straight stem.

### Table 2. Length of the Internodes of the Inflorescence Stems

<table>
<thead>
<tr>
<th>Metamer</th>
<th>Wild Type (mm)</th>
<th>zig (mm)</th>
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<td></td>
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<td>8.0 ± 1.0</td>
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<td>Second internode</td>
<td>11.8 ± 0.8</td>
<td>9.5 ± 0.6</td>
</tr>
<tr>
<td>Third internode</td>
<td>11.6 ± 0.7</td>
<td>11.9 ± 0.5</td>
</tr>
</tbody>
</table>

The inflorescence stems were used after growth had been completed.

a $n = 26$.

b $n = 36$.

c The internodes were numbered from the proximal side of the rosette.
SNAREs are key players in directing membrane fusion processes (McNew et al., 2000; Wickner and Haas, 2000). They can be divided into vesicle SNAREs (v-SNAREs, on vesicle membranes) and target SNAREs (t-SNAREs or syntaxins, on target membranes). The Arabidopsis Genome Initiative has not only confirmed the existence of orthologs of yeast SNAREs in Arabidopsis but also has revealed multiple paralogs and novel groups (Arabidopsis Genome Initiative, 2000; Sanderfoot et al., 2000). Some of these may play roles that are specific for higher plants. In fact, investigation of the mutant of the syntaxin-type SNARE gene KNOLLE showed this gene functions in cytokinesis (Lukowitz et al., 1996; Lauber et al., 1997). However, most of the SNARE disruption mutants examined so far are lethal; therefore, their specific functions have remained unknown (Sanderfoot et al., 2001).

We found that ZIG is identical to AtVTI11, which most likely is a v-SNARE and was cloned as a yeast VTI1 homolog by an Arabidopsis EST database search by Zheng et al. (1999). Yeast Vt1p is involved in multiple vesicle transport pathways, such as transport from the Golgi apparatus to vacuoles via endosomes (Holtzhuis et al., 1998; von Mollard and Stevens, 1999) and retrograde transport within the Golgi apparatus (Lupashin et al., 1997; von Mollard et al., 1997). ZIG/AtVTI11 is expressed in all Arabidopsis organs examined (data not shown). Immunoelectron microscopy has shown that ZIG/AtVTI11 is localized in the trans-Golgi network and the prevacuolar compartment (PVC) in Arabidopsis root cells (Zheng et al., 1999). ZIG/AtVTI11 can substitute for Vti1p in the Golgi-to-vacuole transport of carboxypeptidase Y in the yeast vti1 temperature-sensitive mutant (Zheng et al., 1999). However, these studies have not clarified the precise function of AtVTI11, although it is supposed that AtVTI11 is a housekeeping gene. In plants, there are two different types of vacuoles: lytic vacuoles and storage vacuoles. Storage vacuoles are unique to plants, and extensive studies have suggested that the molecular mechanism by which proteins are transported to plant lytic vacuoles may be more complex than that in yeast (Bassham and Raikhel, 2000). The PVC, which probably is a target for AtVTI11-containing vesicles, seems to mediate traffic to lytic vacuoles.

By characterizing the zig mutant, we have revealed novel function(s) of ZIG/AtVTI11. The zig mutant was morphologically anomalous and showed abnormal gravitropism in its hypocotyl and stem (Figures 1 and 5). These observations indicate that ZIG is involved in both the gravitropic response and plant morphogenesis and suggest that vesicle transport processes may be involved in gravitropism. It has been shown that ZIG/AtVTI11 interacts with the syntaxin homologs AtPEP12 and AtVAM3, which are localized in the PVC and the PVC/vacuole, respectively (Sato et al., 1999; Sanderfoot et al., 1999; Zheng et al., 1999). The mutant zig-3, whose gravitropic response is the most weakly affected of the three zig mutants, has an amino acid substitution in the conserved residue of the coiled-coil domain that is conserved in the VTI1 family. This domain should be involved in

**ZIG/SGR4 Is a SNARE, AtVTI11**

ZIG encodes a member of the SNARE class of proteins. SNAREs are key players in directing membrane fusion pro-

![Image](https://via.placeholder.com/150)

**Figure 7.** Histological Analysis of the zig Mutant. (A) and (C) Epidermal cell layers of wild-type (A) and zig-1 (C) fluorescence stems. (B) and (D) Longitudinal sections of wild-type (B) and zig-1 (D) fluorescence stems. Bars = 50 μm.
interaction with a partner SNARE, and this interaction may decrease in zig-3. It is conceivable that it is the cargo transported by the ZIG-dependent pathway, rather than ZIG itself, that is required for gravitropism or morphogenesis. Notably, although the disruption of VT1 is lethal in yeast (von Mollard and Stevens, 1999), ZIG is dispensable in Arabidopsis. Because Arabidopsis has three VT1 homologs (Arabidopsis Genome Initiative, 2000; Sanderfoot et al., 2000), they may be redundant, at least for the vital role(s) they mediate.

How Do SGR2 and ZIG Relate to Each Other?

Because SGR2 was predicted to have a transmembrane domain, it is expected to localize to a certain membrane compartment, although it does not appear to contain localization signals that would direct it to particular organelles. Interestingly, the cells of some sgr2 embryos have been observed to have abnormal membrane structures (T. Kato and M. Tasaka, unpublished data). Such abnormalities may be responsible for the aberrant morphology seen in some sgr2 mutant seedlings. In addition, it has been reported that phospholipase A1 activity was detected in the vacuole membrane fraction of Acer pseudoplatanus cultured cells (Tavernier and Pugin, 1995). With regard to ZIG, the functional complementation of ZIG/AtVT11 in yeast and the subcellular localization and molecular interactions of this protein in root cells of Arabidopsis suggest that ZIG is involved in vesicle transport to vacuoles, probably via the PVCs (Zheng et al., 1999). Together, these observations suggest that both SGR2 and ZIG participate in vacuole function. In the accompanying paper, the involvement of SGR2 and ZIG in vacuolar functions is investigated.

Figure 8. Molecular Cloning of the ZIG Gene.

(A) Initial mapping placed the zig mutation between the nga76 and DFR markers. Using newly generated PCR markers, the position of the ZIG locus was narrowed to an ~380-kb region. The numbers of recombinants among 2422 chromosomes tested are indicated (recombinant chromosomes/analyzed chromosomes). DNA gel blot analysis using the K16M23 TAC clone as a probe showed that deletions had occurred in both zig-1 and zig-2 in the region that contains MUL8.15.

(B) The structure of the ZIG gene and the mutation sites of the zig alleles examined. Boxes represent exons: closed boxes, translated regions; open boxes, untranslated regions. zig-2 had lost the ZIG gene completely as well as some neighboring genes (data not shown).

(C) Molecular lesion in each allele shown on the protein. The X shows the chromosomal discontinuity in zig-1. Black and gray boxes indicate a predicted transmembrane domain and a coiled-coil domain, respectively, of ZIG/AtVT11.

(D) and (E) Complementation of zig by the wild-type ZIG genomic region. The 5-kb genomic DNA fragment that includes MUL8.15 (A) was transformed into zig-1 plants.

(D) The resulting 4-week-old transgenic plants.

(E) Plants placed horizontally for 2 hr at 23°C in the dark. The arrow indicates the direction of gravity (g).
METHODS

Plant Materials and Growth Conditions

The Columbia (Col) and Wassilewskija (Ws) ecotypes of Arabidopsis thaliana were used as the wild type. sgr2-1, -2, -3, -4, and -6 were isolated from the M2 population of Col that had been mutagenized by ethyl methanesulfonate. sgr2-7 was isolated from T-DNA insertion Ws lines (DuPont) (Fukaki et al., 1996b). sgr2-8, sgr2-9, and sgr2-10 were isolated from the M2 population of Col mutagenized by fast neutron exposure (Lehle Seeds, Round Rock, TX). zig-1/sgr4-1 was isolated from fast neutron–mutagenized Col seed lots, as described previously (Yamauchi et al., 1997). zig-2 and zig-3 were newly isolated from fast neutron–mutagenized Col seed and T-DNA insertion Ws lines (DuPont), respectively. The screening strategy used to isolate these mutants has been described (Fukaki et al., 1996b), and their allelism was confirmed by being crossed with each other. Plants were grown in soil under constant white light at 23°C and used to assay the gravitropism of their inflorescence stems. Three-day-etched seedlings grown on Murashige and Skoog (1962) medium at 23°C also were analyzed as described previously (Fukaki et al., 1996a, 1996b). Approximately 700 seed from 10 siliques were plated on Murashige and Skoog plates and incubated under constant white light at 23°C to analyze the phenotype of the sgr2 seedlings.

Gravitropism Assay

To examine the gravitropic responses of inflorescence stems, intact plants or segments of young primary stems 4 to 8 cm in length were used. The 4-cm-long stem segments included shoot apices and all lateral organs. The stem segments were preincubated in a vertical orientation under light at 23°C and then placed horizontally in darkness at 23°C, as described previously (Fukaki et al., 1996a). To measure the gravitropic response of intact stems, vinyl pots in which the plants were grown were placed horizontally in darkness at 23°C. The curvature of the stem was measured as the angle formed between the growing direction of the apex and the horizontal baseline. At least 20 individuals of each genotype were examined.

Histological Analysis

Stem segments were cut from primary inflorescence stems that grew upright after bolting and fixed in 10% (v/v) formaldehyde, 5% (v/v) acetic acid, and 50% (v/v) ethanol in 0.2-mL tubes under vacuum. After fixation, samples were dehydrated by a series of ethanol washes and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer’s instructions. Sections (3 μm) were stained with toluidine blue. Epidermal cell layers were peeled from the inflorescence stems and then stained with toluidine blue.

Mapping and Cloning of the SGR2 Gene

The sgr2-1 mutation was mapped to lie between the UFO (UNUSUAL FLORAL ORGANS) cleaved-amplified polymorphic sequence marker (5 recombinants) and the tfl1 genetic marker (35 recombinants) on chromosome 1 after analysis of 600 chromosomes. One of five recombinants in the UFO-sgr2 region had a recombinant point in the m402 restriction fragment length polymorphism marker sgr2. A bacterial artificial chromosome (BAC) and transformation-competent artificial chromosome (TAC) contig between UFO and m254 was constructed. A TAC library (Liu et al., 1999) was screened using various DNA probes (UFO, m402, and m254), and BAC clones were selected on the basis of World Wide Web–derived information on BAC end sequences and BAC hybridization data on yeast artificial chromosome (YAC) and BAC fingerprints. Some polymerase chain reaction (PCR) markers were synthesized on the basis of the end sequences of the clones obtained. Additional fine analysis of 1100 chromosomes (total, 1700 chromosomes) was performed. sgr2 was mapped initially between the AtSO392 simple sequence length polymorphism marker (42 recombinants) and the T27K12-SP6 simple sequence length polymorphism marker (116 recombinants).

DNA gel blot analysis was performed to determine the polymorphism between the wild-type and the fast neutron–mutagenized sgr2-8, sgr2-9, and sgr2-10 genomic DNA. Thus, 2 μg of genomic DNA was digested with eight restriction enzymes: XbaI, HindIII, BglI, DraIII, EcoRI, EcoRV, KpnI, and PstI (data not shown). The >10-kb XbaI fragment (Figure 5A, probe X) digested from BAC F11B17 was used as a probe. Hybridization and signal detection were performed with the ECL Direct System (Amersham Pharmacia Biotech).

The 26-kb genomic DNA region, including both sides of the XbaI fragment, was sequenced. Eight putative genes were identified in the genome using the BLAST search and the annotation of the Arabidopsis Genome Initiative. By sequencing each putative coding region, each mutation of all 10 sgr2 alleles was mapped to one gene. 5′- and 3′-rapid amplification of cDNA ends kits from Life Technologies (Grand Island, NY) defined the coding region. A 7.3-kb BamHI–ClaI fragment that included the 1.2-kb upstream region of the gene and the 500-bp downstream region was cloned into pBIn19. The clone was introduced into Agrobacterium tumefaciens strain MP90 and transformed into the sgr2-1 plants by the floral dip method (Clough and Bent, 1998).

Mapping and Cloning of the ZIG Gene

The zig-1 homozygous mutant was crossed to Landsberg erecta wild-type plants to generate a mapping population. Approximately 50 recombinants of the F2 progeny were screened for the zig-1 phenotype for rough mapping. The position of ZIG was located in the middle of chromosome 5. For fine scale mapping, DNA was prepared from 1211 F2 progeny. Polymorphism between Col and Landsberg erecta were identified, which allowed PCR markers to be designed based on the chromosome 5 sequence data from the Arabidopsis Genome Initiative supplied by the Kazusa group. The resulting cleaved-amplified polymorphic sequence markers were used to map the recombination breakpoints by PCR and restriction digestion.

The 5.0-kb genomic DNA fragment from TAC clone K16M23 (Liu et al., 1999) was cloned into binary vector pBIN19. The construct (pBINzigZIG) was transformed into A. tumefaciens strain MP90 and then introduced into the zig-1 plants (Clough and Bent, 1998). T1 plants were selected by resistance to kanamycin and stem shape. The presence of the transgene in these plants was tested by PCR. Segregation of the transgene in the T2 generation was confirmed.
RNA Gel Blot Analysis

For RNA gel blot analysis, total RNA was isolated according to methods using aurintricarboxylic acid. Subsequently, 5 μg of total RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech). The 700-bp SGR2 cDNA fragment amplified by PCR with primers 5'-GATTTTGTGACAGTTTCACAG-3' and 5'-ACTGATCTATTCTGACAGG-3' was cloned into pGEM-T Vector (Promega) and used as a template for RNA probes. Blots were hybridized overnight at 23°C in the hybridization buffer containing 1 μg/mL digoxigenin-labeled RNA probe. Immunological detection was performed according to the manufacturer's instructions. Chemiluminescence was performed with CDP-Star (Boehringer Mannheim) and exposed to Hyperfilm MP (Amersham Pharmacia Biotech).

Accession Numbers

The GenBank accession numbers for SGR2 and Zig are AB073133 and AF114750, respectively.

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REFERENCES


SGR2, a Phospholipase-Like Protein, and ZIG/SGR4, a SNARE, Are Involved in the Shoot Gravitropism of Arabidopsis
Takehide Kato, Miyo Terao Morita, Hidehiro Fukaki, Yoshiro Yamauchi, Michiko Uehara, Mitsuru Niihama and Masao Tasaka

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