Plant Physiology, March 2000, Vol. 122, pp. 695–704, www.plantphysiol.org © 2000 American Society of Plant Physiologists

A Novel Gibberellin-Induced Gene from Rice and Its Potential Regulatory Role in Stem Growth[1]

Esther van der Knaap[2], Jeong Hoe Kim, and Hans Kende*

Michigan State University-Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824–1312

***Os-GRF1* (*Oryza sativa-GROWTH-REGULATING FACTOR1*) was identified in a search for genes that are differentially expressed in the intercalary meristem of deepwater rice (*Oryza sativa* L.) internodes in response to gibberellin (GA). Os-GRF1 displays general features of transcription factors, contains a functional nuclear localization signal, and has three regions with similarities to sequences in the database. One of these regions is similar to a protein interaction domain of SWI2/SNF2, which is a subunit of a chromatin-remodeling complex in yeast. The two other domains are novel and found only in plant proteins of unknown function. To study its role in plant growth, *Os-GRF1* was expressed in Arabidopsis. Stem elongation of transformed plants was severely inhibited, and normal growth could not be recovered by the application of GA. Our results indicate that Os-GRF1 belongs to a novel class of plant proteins and may play a regulatory role in GA-induced stem elongation.**

Plants adjust their final height to the prevailing environmental conditions by increasing or decreasing their growth rate in response to external and internal signals. To increase yield, the height of crop plants is often manipulated either genetically or by application of plant growth regulators. GA plays a major role in regulating stem or internodal elongation, as evident from physiological studies and from the phenotype of mutants impaired in GA biosynthesis or perception (Hooley, 1994; Swain and Olszewski, 1996; Ross et al., 1997; Harberd et al., 1998). Despite its complexity, the GA biosynthetic pathway has been well characterized (Hedden and Proebsting, 1999). In contrast, much remains to be learned about the GA signal transduction pathway that leads to stem elongation and other GA-regulated processes. Genetic analysis of GA-response mutants of Arabidopsis and cloning of the respective genes led to the identification of GA signal transduction components that appear to be negative regulators of GA action. Two putative transcription factors with high sequence similarity to each other, GAI (Peng et al., 1997) and RGA (Silverstone et al., 1998), were shown to mediate responses to GA, and their functions appear to be partially overlapping (Harberd et al., 1998; Silverstone et al., 1998). Overexpression of *SHI*, which also encodes a putative transcription factor, led to a semi-dominant dwarf phenotype (Fridborg et al., 1999). The product of the *SPY* gene shows significant similarity to O-linked GlcNAc transferases of animals and may regulate the activity of proteins of the GA signaling pathway by post-translational modifications (Jacobsen et al., 1996; Thornton et al., 1999).

Identification of genes whose expression is controlled by GA complements characterization of GA response mutants as an approach to elucidate the mode of action of GA. Genes whose transcript levels change following treatment with GA may encode proteins that act downstream of the GA signal transduction pathway and that may play a crucial role in regulating growth. One group of such genes encodes enzymes of GA biosynthesis and metabolism. As part of a negative feedback mechanism, the expression of these genes changes in response to GA (Hedden and Proebsting, 1999; Thomas et al., 1999). Whereas several GA-regulated genes of the cereal aleurone layer have been studied in detail (Bethke et al., 1997), relatively few GA-controlled genes have been identified in elongating stems. They include genes in tomato (Shi et al., 1992; Jacobsen and Olszewski, 1996), pea (Wu et al., 1993), and Arabidopsis (Phillips and Huttly, 1994). The function of these genes in GA-regulated stem elongation, however, is unknown.

Deepwater rice (*Oryza sativa* L.) is grown in Southeast Asia and has the capacity to elongate very rapidly when it is inundated by flood waters during the monsoon season (Kende et al., 1998). This growth response is based on internodal elongation and results at least in part from an ethylene-mediated increase in the ratio of an endogenous growth promoter (GA) and growth inhibitor (abscisic acid [ABA]) (Hoffmann-Benning and Kende, 1992). The ultimate growth-promoting hormone in internodes of deepwater rice is GA (Raskin and Kende, 1984), and the primary site of GA action is the intercalary meristem at the base of the growing internode (Sauter et al., 1993). Applied GA increases the rate of cell production in the intercalary meristem and promotes the growth of cells to three times their normal length in the internodal elongation zone (Raskin and Kende, 1984; Sauter and Kende, 1992; for the anatomy of the rice stem, see Fig. 3E). Unlike the shoot apical meristem of dicotyledoneous plants, from which growth of the stem and the formation of leaves and floral organs originate, the intercalary meristem of grasses contributes to stem elongation only. Therefore, GA-induced stem growth

[1] This work was supported by the National Science Foundation (grant no. IBN9722915) and by the U.S. Department of Energy (grant no. DE–FG02–91ER20021).

[2] Present address: Department of Plant Breeding and Biometry, Cornell University, Ithaca, NY 14853–1673.

* Corresponding author; e-mail hkende@pilot.msu.edu; fax 517–353–9168.

can be investigated in rice without interference by other developmental processes that occur in the shoot apical meristem.

Several GA-regulated genes have been identified in deepwater rice internodes. They can be grouped into three categories: (a) genes whose products function in the cell cycle; (b) genes encoding proteins that act as cell wall-loosening factors; and (c) genes whose role in stem growth is unknown (Kende et al., 1998; Van der Knaap, 1998). We report here the identification of a novel gene, *Os-GRF1* (*Oryza sativa-GROWTH-REGULATING FACTOR1*), whose expression increases rapidly in the intercalary meristem of deepwater rice internodes in response to GA and submergence. The protein encoded by *Os-GRF1* shares features common to transcription factors and transcriptional activators. Significant amino acid sequence similarity has been found to hypothetical plant proteins in the Arabidopsis genome database and to proteins encoded by plant cDNAs. To investigate its role in GA-mediated growth, *Os-GRF1* was expressed in Arabidopsis. Stem elongation of the transformed plants was greatly inhibited and was not reversed by the application of GA. Our results indicate that *Os-GRF1* encodes a novel plant-specific protein that may be involved in transcriptional regulation and in mediating GA-induced stem elongation.

MATERIALS AND METHODS

Plant Material

Seeds of deepwater rice (*Oryza sativa* L. cv Pin Gaew 56) were obtained from the International Rice Research Institute (Los Baños, Philippines). Submergence experiments were conducted with 12-week-old plants according to the method of Métraux and Kende (1983). For treatment with GA, 20-cm-long stem sections containing the growing internode were excised and placed into a 50 μM GA_3 solution (Raskin and Kende, 1984). Incubation was allowed to proceed for the periods indicated, and then the intercalary meristem was excised, frozen immediately, and stored at −80°C until use. Seeds of Arabidopsis ecotype Columbia were provided by Dr. Pamela Green and Linda Danhoff (Michigan State University).

Identification of *Os-GRF1* and *D40170*

A differentially displayed 222-bp cDNA fragment was identified using the primers T12MG and OPA04 (Van der Knaap and Kende, 1995). A full-length cDNA, *Os-GRF1*, was obtained by screening an intercalary-meristem-specific cDNA library with the differentially displayed product. Sequence analysis was performed at the W.M. Keck Facility, Yale University, New Haven, CT. The sequences were aligned using Sequencher, version 3.0 (Gene Codes Corporation, Ann Arbor, MI). The rice expressed sequence tag (EST) *D40170*, whose deduced amino acid sequence shows similarity to Os-GRF1, was obtained from the National Institute of Agrobiological Resources, Tsukuba, Japan. Sequence and RNA gel-blot analysis of *D40170* indicated that it was a partial cDNA.

Nuclear Localization of Os-GRF1

To facilitate subcloning of *Os-GRF1*, restriction enzyme sites were introduced by PCR with Pwo polymerase (Boehringer) using the following primers: 5′-TCGGTCT-AGAGGCGGTCGGTCGACGCTGAA-3′ and 5′-TCATTG-TGGATCCGGGAGGTGGTGGTGATC-3′. The PCR product corresponded to the entire coding region of *Os-GRF1*, except for the bases encoding the last five amino acids. This PCR product was inserted into *pMF6*, a monocot-specific transient assay transformation vector, in frame with the coding region of the reporter protein β-glucuronidase (GUS), yielding the *pOs-GRF1::GUS* construct (Varagona et al., 1992). Onion epidermal cells were transformed and nuclear localization of GUS was assayed according to the method of Varagona et al. (1992).

RNA Gel-Blot Analyses

Total RNA was isolated according to the method of Puissant and Houdebine (1990). Twenty micrograms of RNA per sample was analyzed by standard RNA gel blotting (Ausubel et al., 1987). The following DNA fragments were used as probes: an insert containing the terminal 737 bp from the 3′ end of *Os-GRF1* (starting at the codon for Gln-318 [see Fig. 1A] and excluding the poly-A tail), and the inserts of *D40170*, of *E37*, and of a partial histone H3 cDNA clone (Van der Knaap and Kende, 1995). Fifty nanograms of template DNA was labeled in the presence of α-[^{32}P]dCTP (3,000 Ci/mmol, NEN Life Science Products, Boston) using a random primer labeling kit (Boehringer Mannheim/Roche, Basel). A RNA probe of *cycOs1*, which contained the 3′ untranslated region and most of the region encoding a mitotic cyclin (Sauter et al., 1995), was prepared in the presence of α-[^{32}P]UTP (800 Ci/mmol, NEN). A RNA probe was also prepared from the 737-bp cDNA fragment of *Os-GRF1* to detect the transcripts in Figure 3C. Hybridization and washing conditions for the RNA gel blots were as described by Van der Knaap et al. (1999). The radioactivity on blots was quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

Genomic DNA Analyses

Rice genomic DNA was isolated from a CsCl gradient according to the method of Ausubel et al. (1987). Arabidopsis genomic DNA was isolated in hot (65°C) hexadecyltrimethylammonium bromide (CTAB) buffer (2% [w/v] CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, and 0.2% [v/v] β-mercaptoethanol), and incubated at 65°C for 30 min. After chloroform extraction, the DNA was precipitated with 0.6 volume of isopropanol. The pellet was dissolved in Tris-EDTA, and RNA was removed by RNase A treatment. After organic extraction, the DNA was precipitated with one-third volume of 7.5 M NH_4-acetate and 2.5 volumes of ethanol, and subsequently dissolved in the appropriate amount of Tris-EDTA. Five micrograms of rice DNA and 500 ng of Arabidopsis DNA were analyzed by standard DNA gel blotting (Ausubel et al., 1987). Hybridization and washing conditions were as described for the

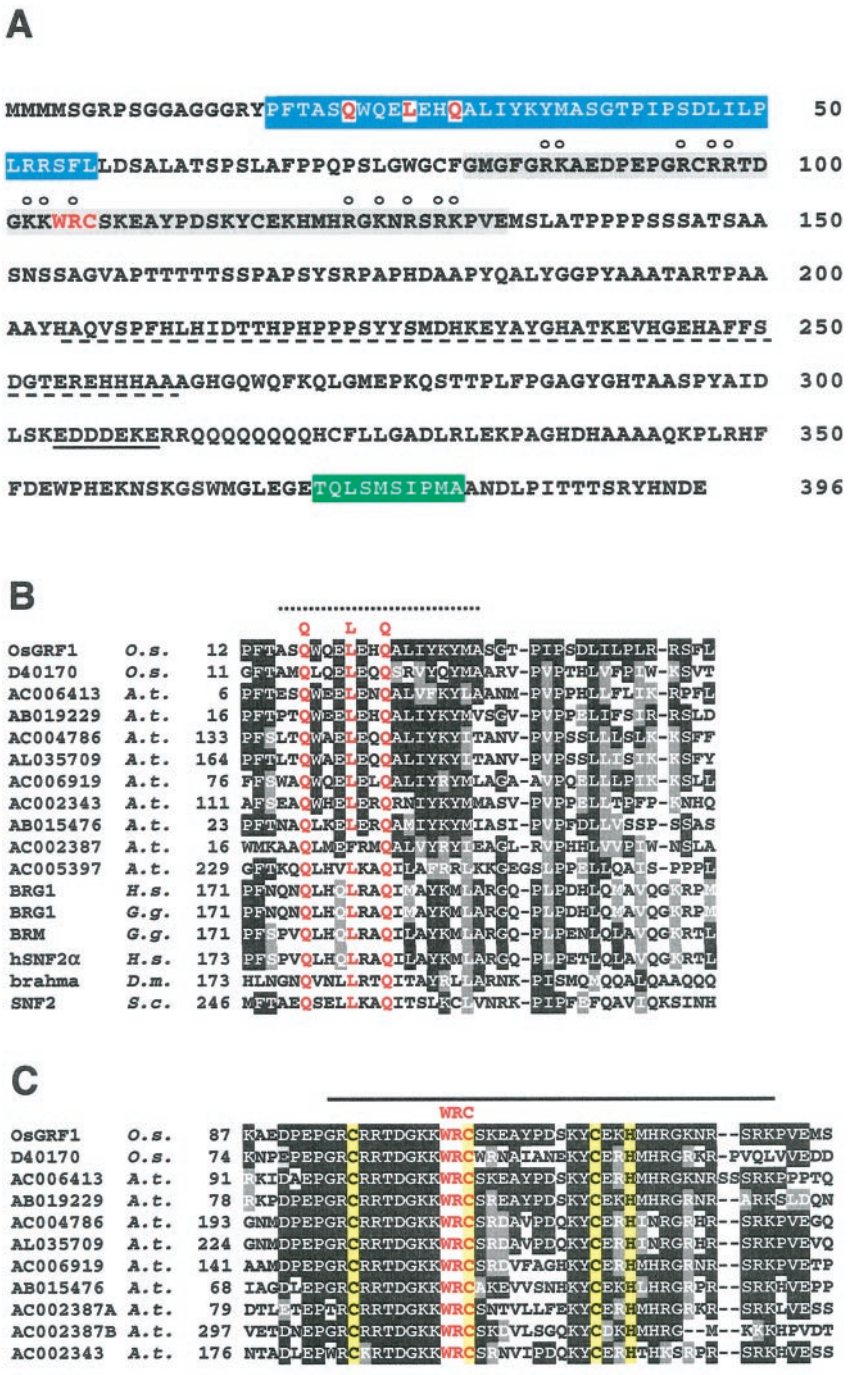

Figure 1. Sequence analysis of Os-GRF1. A, Amino acid sequence of Os-GRF1. The QLQ domain is boxed in blue, the WRC domain in gray, and the TQL domain in green. The His-rich region is underlined with dashes, and the acidic region with a solid line. In the WRC domain, amino acids that may be involved in nuclear localization are indicated by circles above the respective letters. B, Alignment of the QLQ domain with that of other organisms (multiple alignment by Clustal W; Thompson et al., 1994; http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html). *O.s.*, *Oryza sativa* (accession no. for Os-GRF1 is AF201895); *A.t.*, Arabidopsis; *H.s.*, *Homo sapiens* (accession nos. for BRG1 and hSNF2α are P51532 and S45251, respectively); *G.g.*, *Gallus gallus* (accession nos. for BRG1 and BRM are X91637 and X91638, respectively); *D.m.*, *Drosophila melanogaster* (accession no. for brahma is P25439); *S.c.*, *Saccharomyces cerevisiae* (accession no. for SNF2 is P22082). The dotted line denotes a region of a predicted α-helix. C, Alignment of the WRC domain by Clustal W. The letters on yellow background comprise the potential metal-binding motif $CX_9CX_{10}CX_2H$. The solid line denotes the region rich in basic amino acids.

hybridization of random-primer-labeled probes to RNA gel blots (Van der Knaap et al., 1999). The 737-bp cDNA fragment of *Os-GRF1* was used as a probe for the rice DNA gel blot, and the full-length *Os-GRF1* cDNA for the Arabidopsis DNA gel blot.

Transformation of Arabidopsis

Os-GRF1 was inserted in sense orientation into the *Xba*I-*Cla*I site of the binary vector *pGA643* (An et al., 1988). For cloning purposes, the construct thus created, *pGA::GRF1*, lacked the 428 bp from the 3′ end of *Os-GRF1*, leaving only 74 bp of its 3′ untranslated region. *Agrobacterium tumefaciens* strain GV3101 (C58C1 Rif^R) pMP90 (Gm^R) was transformed with *pGA::GRF1* or *pGA643*. *A. tumefaciens* containing *pGA::GRF1* or *pGA643* were vacuum infiltrated into Arabidopsis ecotype Columbia or ecotype Landsberg *erecta*, as described by Van Hoof and Green (1996). The seeds collected were surface-sterilized and plated on Murashige and Skoog (MS) medium (Life Technologies/Gibco-BRL, Cleveland) containing 1% (w/v) Suc, 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES), 0.8% (w/v)

phytagar (Life Technologies/Gibco-BRL), 500 μg/mL vancomycin, and 50 μg/mL kanamycin (Sigma). After 2 weeks, transgenic plants were transferred to soil and grown in a growth chamber at 20°C in 16-h light (100 μmol m^{-2} s^{-1})/8-h dark cycles. The severe female sterile lines were maintained by backcrossing to wild-type Arabidopsis using the transgenic plants as male parents.

Experiments with Transgenic Plants

Seeds were imbibed and kept for 4 d at 4°C in the dark. They were allowed to germinate on MS plates containing 50 μg/mL kanamycin and grown, unless otherwise indicated, under the conditions described above. The germination times of line 13 and of two control transformed lines were measured with 50 to 110 seeds each per MS plate (n = 6) in a growth chamber as described above. To determine growth of the hypocotyl, seeds were germinated on MS plates placed in a slanted position under continuous light (100 μmol m^{-2} s^{-1}) at 25°C. After 3 d, the length of the hypocotyls was measured in increments of 0.25 mm using a dissecting microscope. Petiole and pedicel lengths were determined 33 d after germination. Petioles of the three longest rosette leaves and the first three pedicels per plant were measured.

RESULTS

Identification of *Os-GRF1*

Using differential display of mRNA, we identified a 222-bp cDNA fragment corresponding to a transcript whose level increased in the intercalary meristem of rice within 2.5 h of treatment with GA (Van der Knaap and Kende, 1995). A rice cDNA library prepared from the intercalary meristem was screened with this cDNA, and a full-length 1,891-bp clone, *Os-GRF1*, was isolated. The predicted 43-kD protein encoded by *Os-GRF1* contains a Pro/Ser/Thr-rich region, a His-rich region, a region rich in acidic residues, a homopolymeric sequence of Gln, and a putative bipartite nuclear localization signal (NLS) (Hicks and Raikhel, 1995) (Fig. 1A). These features are reminiscent of transcription factors (Mitchell and Tjian, 1989; Gerber et al., 1994). Database searches indicated weak similarities to a range of transcription factors, transcriptional activators, and homeo-domain proteins. Using the BLASTP program and the NR database (Altschul et al., 1997), we found the highest similarity to several hypothetical proteins in the Arabidopsis genome database. The highest similarity to known proteins was to the chicken BRM and BRG1 and the human BRG1 and SNF2α proteins (Fig. 1B). These proteins are related to yeast SWI2/SNF2, a subunit of a large chromatin-remodeling complex (Peterson and Tamkun, 1995).

Three regions of Os-GRF1 showing similarities to sequences in the database have been identified (Fig. 1A). The first region close to the N terminus contains the motif QX_3LX_2Q and will be referred to as the QLQ domain. A region following the QLQ domain contains the sequence WRC and will be called the WRC domain. The third region close to the carboxyl end will be referred to as the TQL domain. The amino acid alignments of the QLQ and WRC domains with the corresponding regions of other proteins are shown in Figure 1, B and C. The QLQ domain has similarities to a domain involved in protein-to-protein interactions in SWI2/SNF2 and related proteins (Treich et al., 1995) (Fig. 1B). The WRC domain shows higher similarity to other proteins than does the QLQ domain (Fig. 1, B and C). It contains a putative NLS and three Cys and one His residues in the sequence $CX_9CX_{10}CX_2H$, which may function in metal binding (Fig. 1, A and C). Both the QLQ and WRC motifs are present in the same plant proteins shown in the alignments of Figure 1, B and C. Regions of lower similarity containing the WRC motif were identified in searches of the Arabidopsis genomic database. However, these hypothetical proteins did not have the QLQ domain (not shown). The TQL domain (Fig. 1A) was also found in

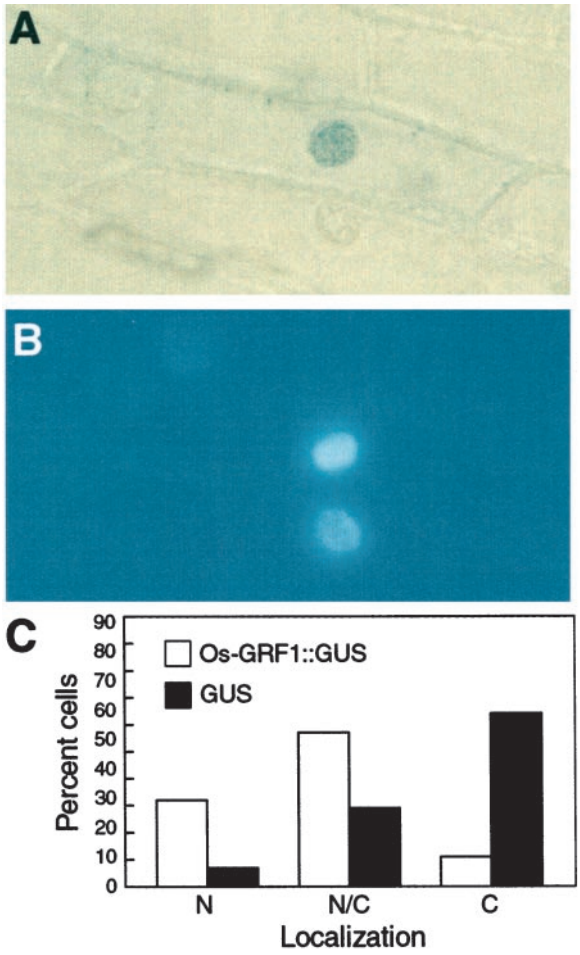

Figure 2. Nuclear localization of Os-GRF1. A, Localization of the Os-GRF1::GUS fusion protein in the nucleus of an onion epidermal cell. The staining reaction was performed with the GUS substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc). B, Staining of the nuclei shown in A with the DNA-specific dye DAPI. C, Localization of Os-GRF1::GUS and of GUS (control) in the nucleus (N), nucleus and cytoplasm (N/C), and cytoplasm (C). Seventy-nine and 240 cells were scored to determine the distribution of Os-GRF1::GUS and GUS, respectively.

two entries of the Arabidopsis database (genomic sequences with accession nos. AC004786, which was also isolated in our cDNA library screen, and AL035709, which is identical to an EST with accession no. N95873; see Fig. 1, B and C). The 10 amino acids of the TQL domain distinguish Os-GRF1 and the proteins encoded by the two above-cited cDNAs from the other proteins containing the QLQ and WRC domains.

Nuclear Localization of Os-GRF1

To address the cellular localization of Os-GRF1, we prepared a construct containing *Os-GRF1* fused in-frame to the reporter gene encoding GUS. This construct was introduced into onion epidermal cells by biolistic bombardment, and the fusion protein was localized by histochemical staining. Blue staining was found to be associated with nuclei (Fig. 2A), a result confirmed by coincident staining with the DNA-specific dye 4′,6-diamino-phenylindole (DAPI) (Fig. 2B). Of the 79 onion cells counted, 32% showed exclusive nuclear localization of the fusion protein, whereas only 7% of the control nuclei contained GUS (Fig. 2C). These results indicate that Os-GRF1 contains a functional NLS.

Expression of *Os-GRF1* and Gene Copy Number in Rice

The transcript level of *Os-GRF1* was determined in growing internodes by RNA gel-blot analysis. The signals were quantified by PhosphorImager analysis and normalized for equal loading relative to the transcript level of *E37*, a gene whose expression did not change significantly over the course of the experiment (Van der Knaap and Kende, 1995) (Fig. 3, A and B). The level of *Os-GRF1* mRNA in the intercalary meristem of stem sections increased more than 4-fold within 3 h of GA treatment and 8-fold within 4 h (Fig. 3, A and B). The transcript level of *D40170*, a rice EST that contains both the QLQ and WRC motifs, did not change in response to GA (Fig. 3A).

The expression pattern of *Os-GRF1* was also investigated in the intercalary meristem of submerged plants (Fig. 3C). *Os-GRF1* mRNA levels increased within 4 h of submergence and reached a maximum after 8 h. The increase in transcript levels of *Os-GRF1* was compared with that of the histone H3 transcript and of the cyclin transcript *cycOs1*. Histone H3 is a marker for the S-phase of the cell cycle (Van der Knaap and Kende, 1995) and *cycOs1* for the entry into the M-phase (Sauter et al., 1995; Sauter, 1997). The signals for the histone H3 and *cycOs1* transcripts were quantified and normalized for equal loading with *E37*. A greater than 3-fold increase in histone H3 transcript level was found 8 h after the start of submergence, and an increase in *cycOs1* mRNA was observed after 16 h. Because the signal for *Os-GRF1* could not be detected by PhosphorImager analysis at the 0-h time point, the *Os-GRF1* signals were not quantified. It is clear, however, that the increase in the expression of the *Os-GRF1* gene preceded expression of the histone H3 and of *cycOs1* genes. This indicates that changes in *Os-GRF1* transcript levels occur during submergence before changes in the cell cycle activity are apparent. A transient decrease in *Os-GRF1* transcript levels was observed reproducibly in both GA-treated stem sections and in submerged plants. The significance of this temporary decline is not known.

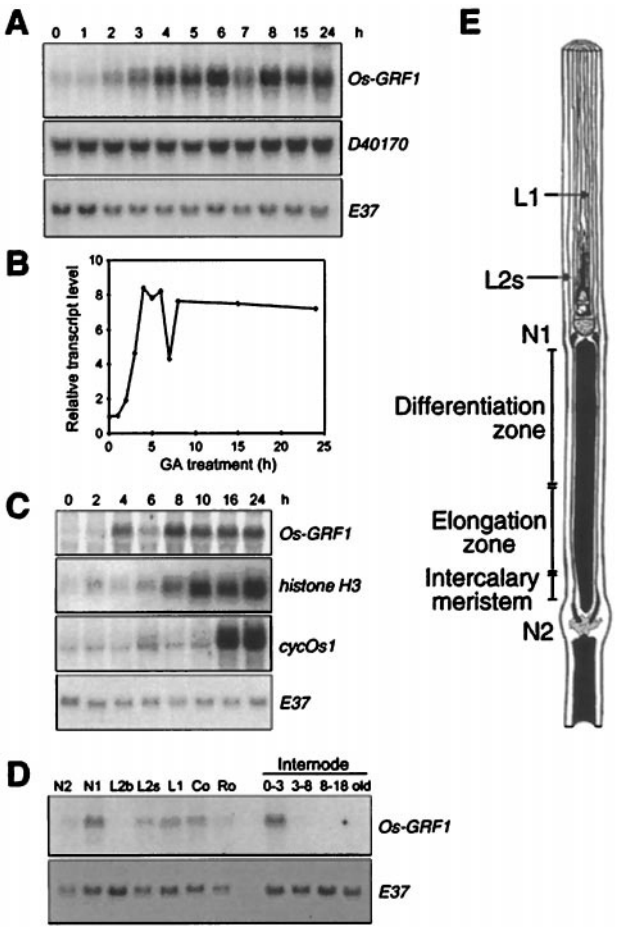

Figure 3. Expression of the *Os-GRF1* gene in rice. A, Rice stem sections were incubated in 50 μM GA_3 for the times indicated above the lanes. Expression of *Os-GRF1* and *D40170*, a rice EST that contains both the QLQ and WRC motifs, was determined in the intercalary meristem by RNA gel-blot analysis (20 μg of total RNA per lane). *E37* served as loading control. B, Quantitative analysis of *Os-GRF1* expression shown in A using a PhosphorImager. The values were normalized to the *E37* loading control. C, Expression of *Os-GRF1* in submerged plants. Whole plants were submerged for the times indicated above the lanes. Expression of the *Os-GRF1*, histone H3, and *cycOs1* genes in the intercalary meristem was determined by RNA gel-blot analysis (20 μg of total RNA per lane). *E37* served as loading control. D, Tissue-specific expression of *Os-GRF1*. N2, Second highest node; N1, highest node containing the shoot apex; L2b, basal 2 cm of second youngest leaf blade; L2s, basal 2 cm of second youngest leaf sheath; L1, youngest leaf; Co, coleoptile 3 d after germination; Ro, root 3 d after germination; 0–3, internodal region 0 to 3 mm above N2 containing the intercalary meristem; 3–8, internodal region 3 to 8 mm above N2 containing the elongation zone; 8–18, internodal region 8 to18 mm above N2 containing the upper part of the elongation zone and the differentiation zone; old, oldest part of the internode. *E37* served as loading control. E, Schematic representation of a rice stem section containing the uppermost internode.

In the internode, the expression of *Os-GRF1* was only detected in the intercalary meristem (Fig. 3D), which is the

primary site of GA action (Sauter et al., 1993). No transcript was found in the elongation zone, in the differentiation zone, or in the oldest part of the internode. High transcript levels were also detected in the shoot apex. Lower expression of *Os-GRF1* was found in the coleoptile and in the youngest leaf. Weak expression was seen in the second-highest node below the growing internode, in the basal part of the second-youngest leaf sheath, and in the root. The anatomy of the rice internode is shown in Figure 3E.

DNA gel-blot analysis was employed to investigate whether genes related to *Os-GRF1* exist in rice. The hybridization probe was derived from the 3′ region of *Os-GRF1* and did not include the regions encoding the QLQ and WRC domains. Three digests produced one predominant and at least one additional fainter band (Fig. 4). The probe used contained a *Pst*I restriction site, which explains the presence of several bands in the lane containing *Pst*I-digested DNA. The results indicate that the rice genome may contain at least one additional gene that hybridizes to the *Os-GRF1* probe at high stringency. The same genomic DNA blot was hybridized to *D40170*, the rice EST that also contains the QLQ and WRC motifs. The banding pattern of the DNA gel blot was different from that obtained with *Os-GRF1* as a probe (results not shown). None of the fainter bands on the blot shown in Figure 4 hybridized to *D40170*.

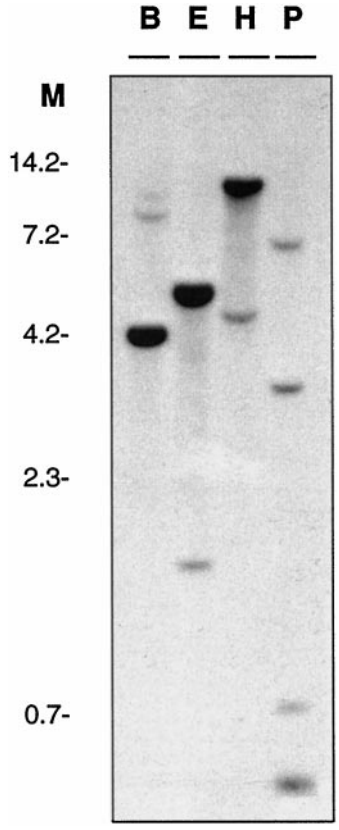

Figure 4. DNA gel-blot analysis of *Os-GRF1*. Rice genomic DNA was digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), or *Pst*I (P). The blot was probed with a random-prime-labeled insert derived from the 3′ 737-bp region of *Os-GRF1*. Lane M, Molecular size markers (kb). The washing conditions were 0.1× SSC, 0.1% (w/v) SDS at 65°C.

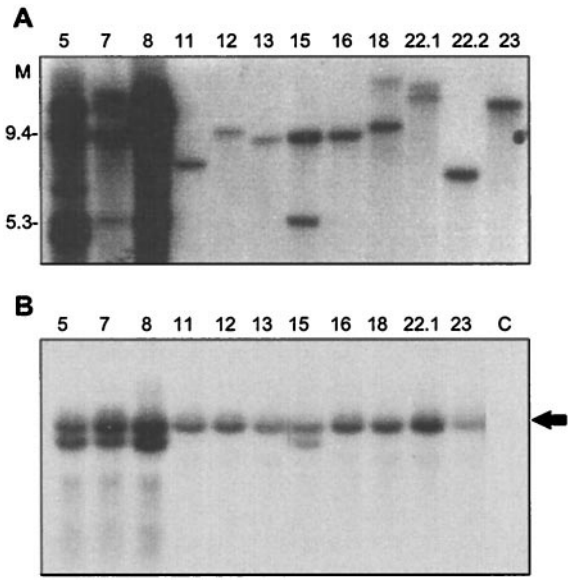

Figure 5. Copy number and expression of *Os-GRF1* in Arabidopsis. A, DNA gel-blot analysis of *Bam*HI-digested DNA from the individual T_1 lines denoted above the lanes. The blot was hybridized with a random-prime-labeled probe derived from the full-length *Os-GRF1* cDNA. B, *Os-GRF1* transcript levels in individual T_2 lines. The RNA gel blot was hybridized with a random-prime-labeled insert derived from the 3′ 737-bp region of *Os-GRF1*. The arrow indicates the position of the full-length *Os-GRF1* mRNA. C, Control plants transformed with the vector alone.

Expression of *Os-GRF1* in Arabidopsis: DNA and RNA Gel-Blot Analyses

To further investigate the function of Os-GRF1 in growth, *Os-GRF1* was expressed in Arabidopsis under control of the cauliflower mosaic virus 35S promoter. The transgenic plants obtained were derived from independent transformation events, as determined by DNA gel-blot analysis (Fig. 5A). In Arabidopsis ecotype Columbia, a total of 17 independent kanamycin-resistant T_1 plants were collected. The phenotype of the transgenic plants is described in the section below. The appearance of more than one band on the DNA gel blot signified the presence of more than one T-DNA insert in the genome. DNA gel-blot analysis of plants with the most severe phenotype (lines 11, 12, 13, and 23) showed a single band, indicating the presence of one copy of the transgene. The severe phenotype of these lines was transmitted to the T_2 generation in a Mendelian fashion. A single band was also observed in the T_1 plants 16 and 22.2, which also showed a severe phenotype. However, no offspring were obtained from line 22.2, and the progeny of line 16 showed variations in phenotype ranging from wild type to severe. The remaining transformed lines contained at least two inserts and showed less severe (lines 18 and 22.1) or wild-type (lines 5, 7, 8, and 15) phenotypes.

The expression of the transgene was determined by hybridizing *Os-GRF1* to RNA isolated from 2-week-old T_2 seedlings transformed with the *Os-GRF1* construct or with the vector alone (Fig. 5B). Equal RNA loading was confirmed by ethidium bromide-staining of the ribosomal

RNA (results not shown). The abundance of the full-length *Os-GRF1* transcript did not correlate with the severity of the phenotype in either the T_1 or the T_2 generations. An additional band corresponding to mRNA of slightly shorter length was only found in plants with an apparent wild-type phenotype (lines 5, 7, 8, and 15). Similar results were obtained with RNA isolated from mature leaves and in experiments using other regions of *Os-GRF1* as a probe (Van der Knaap, 1998). Thus, the copy number of the transgene was inversely correlated with the stability and severity of the phenotype, whereas the transcript level was not correlated to the severity of the phenotype. The phenotype of plants from line 13 is described below. The same phenotype was observed in T_1 and T_2 plants of lines 11, 12, and 23 and in T_1 plants of lines 16 and 22.2.

The Phenotype of Arabidopsis Plants Expressing *Os-GRF1*

The most striking phenotype of plants expressing *Os-GRF1* was the development of flowers inside the rosettes without concomitant stem elongation (Fig. 6, A and B). Control plants transformed with the vector alone had elongated inflorescence stems 7.5 weeks after germination, while stems of plants transformed with *Os-GRF1* had barely started to elongate by that time (Fig. 6B). Whereas wild-type Arabidopsis plants stopped growing approximately 8 weeks after germination, the lateral shoots of plants expressing *Os-GRF1* continued to grow for another 3 months to a final height that was one-half that of wild-type plants (Fig. 6C). The growth habit was atypical, however, showing severely reduced apical dominance (Fig. 6C) and, occasionally, fasciated and bifurcated stems (Van der Knaap, 1998). Plants expressing *Os-GRF1* also had curly rosette leaves (Fig. 6A) and altered gynoecia. The carpels were only partially fused, the septum dividing the ovule was missing, and the stigma surface was sometimes replaced by a leaf-like outgrowth. The plants exhibited female sterility and reduced male fertility (Van der Knaap, 1998).

The timing of germination, flowering, and stem growth was compared between control plants and plants expressing *Os-GRF1* (Table I). In *Os-GRF1* transformants, germination was delayed by 19 h, average flowering time by 3 d,

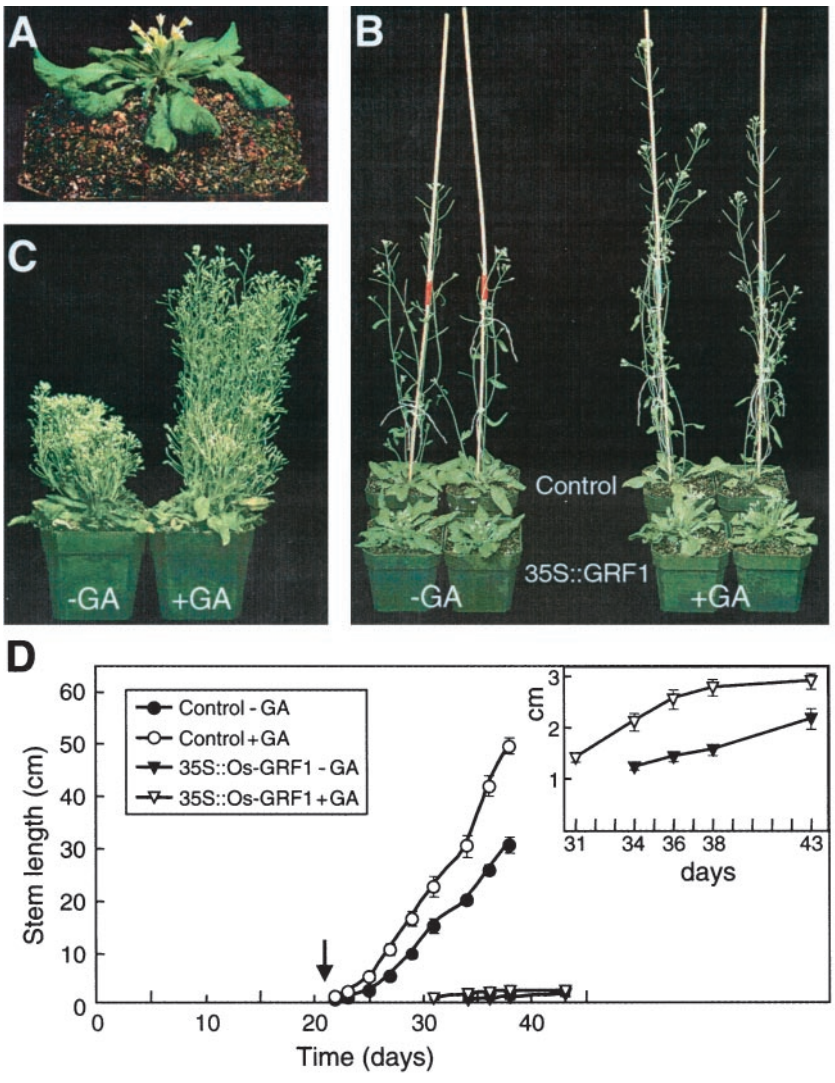

Figure 6. Growth of Arabidopsis plants expressing *Os-GRF1* (Line 13). A, Five-week-old plants that flowered without concomitant bolting and showed curly leaves. B, GA_3 (1 μg) was applied in a 25-μL drop of a 0.01% (v/v) Tween 20 solution to the center of rosettes of 3-week-old plants transformed with *Os-GRF1* or with the vector alone (control) before bolting of the control plants had started. This treatment was repeated three times. For the minus GA treatment, GA_3 was omitted from the Tween 20 solution. The photograph was taken when the plants were 7.5 weeks old. C, Plants transformed with *Os-GRF1* were sprayed with 0.02% (v/v) Tween 20 solution with or without 50 μM GA_3 beginning 21 d after germination and then twice a week for 22 d. The plants were grown for an additional month without further hormone treatment. The primary stem had died off when the photograph was taken, but secondary shoots had continued to grow. D, Growth curve of the primary stem of plants (n = 7) treated with GA_3 as described in C. The arrow denotes the start of hormone treatment, which was repeated twice a week for 17 and 22 d for the control plants and for plants expressing *Os-GRF1*, respectively. The growth of control plants was measured until d 38 after germination; that of the plants transformed with *Os-GRF1* until d 43. The inset is a magnification of the growth curve for the primary stem of *Os-GRF1*-expressing plants treated with and without GA.

Table I. *Germination, flowering, and bolting time of Arabidopsis transformed with 35S::Os-GRF1 or with the vector alone (control)*

Developmental Process	Control	*35S::Os-GRF1*
Germination[a]	64.1 (0.8)[d]	82.7 (1.5)
Flowering[b]	21.7 (1.2)	24.3 (3.6)
Bolting[c]	22.6 (1.2)	35.9 (1.9)

[a] Germination time was measured in hours from the time the seeds were placed in the growth chamber until 90% of the seeds had green, open cotyledons. [b] Flowering time was measured in days from the time of germination until the first flower had opened. [c] Bolting time was measured in days from the time of germination until the inflorescence stem had elongated by 1 cm. [d] The nos. in parentheses denote the SE.

and the onset of stem growth by 13 d. Even though the long delay in stem growth was the most conspicuous result of *Os-GRF1* expression, developmental processes in general appeared to lag. *Os-GRF1* was also expressed in Arabidopsis ecotype Landsberg *erecta*. The phenotype was similar to that observed in the Columbia background, but was in some transformants so severe that stem elongation and flower development never occurred (results not shown).

The Effect of GA on Growth of Arabidopsis Plants Expressing *Os-GRF1*

Because *Os-GRF1* was identified in a screen for GA-induced genes in rice internodes, we investigated the effect of applied GA on several growth processes in Arabidopsis plants that expressed *Os-GRF1*. GA promoted elongation of the inflorescence stem in plants expressing *Os-GRF1* slightly, while enhancing stem growth in control plants considerably (Figs. 6, B and D). Thus, applied GA could not restore growth of the primary stem of plants expressing *Os-GRF1* to that of control plants. However, in older *Os-GRF1*-expressing plants, which show greatly reduced apical dominance, GA promoted elongation of lateral shoots markedly (Fig. 6C).

We investigated the effect of *Os-GRF1* expression and GA on growth processes other than stem elongation (Table II). In plants expressing *Os-GRF1*, the hypocotyl was slightly longer than in control plants but growth of the petiole and pedicel was not significantly affected. The promotion of hypocotyl elongation by GA was comparable in control plants and in plants expressing *Os-GRF1*, and GA-induced pedicel growth might have been even greater in plants expressing *Os-GRF1* than in the control plants (Table II). Applied GA increased the length of the petiole to the same extent in plants expressing *OsGRF1* and in control plants (Table II). Treatment with GA did not reduce fasciation of the stem and did not ameliorate defects in apical dominance and floral development (Van der Knaap, 1998).

DISCUSSION

Os-GRF1 Is the Prototype Member of a Novel Class of Plant-Specific Genes

We identified a novel plant-specific gene, *Os-GRF1*, whose transcript level increases in internodes of deepwater rice in response to GA and submergence. The protein encoded by *Os-GRF1* has three regions with similarities to sequences in the database. Of these, the N-terminal QLQ domain is the only one with similarity to known proteins, the highest similarity being to proteins related to SWI2/SNF2 of yeast. SWI2/SNF2 is a subunit of a 2-MD, ATP-dependent chromatin-remodeling complex (Peterson and Tamkun, 1995). The similarity between the N-terminal region of yeast SWI2/SNF2 and the corresponding *Drosophila*, human, and chicken proteins is restricted to two short sequences called domain 1 and 2 in the *Drosophila* ortholog brahma (Tamkun et al., 1992). The QLQ domain of Os-GRF1 corresponds to domain 1, which in yeast is responsible for interaction with SNF11, another component of the SWI/SNF complex (Treich et al., 1995). The similarity of the QLQ domain of Os-GRF1 to domain 1 of SWI2/SNF2 indicates that it too may be involved in protein-to-protein interactions.

The WRC domain has features that indicate a function in transcriptional control and DNA binding. The Os-GRF1::GUS fusion protein is targeted to the nucleus of onion epidermal cells, most likely by a NLS that is located in the WRC domain. The WRC domain also contains Cys and His residues in the sequence $CX_9CX_{10}CX_2H$, a motif that may be involved in metal binding. A large class of metal-binding proteins chelates zinc (Berg and Shi, 1996; Borden and Freemont, 1996). These zinc-binding domains or zinc fingers are often present in transcription factors, where they may be involved in DNA binding. The amino acid sequence of the WRC domain and the spacing of the Cys and His residues do not fall into any of the known classes of zinc fingers (Berg and Shi, 1996; Borden and Freemont, 1996). However, HRT, a barley transcriptional repressor protein, has recently been shown to contain three novel

Table II. *Elongation of the hypocotyl, petiole, and pedicel in Arabidopsis transformed with 35S::Os-GRF1 or with the vector alone (control)*

Organ	Control		*35S::Os-GRF1*	
	−GA	+GA	−GA	+GA
Hypocotyl[a]	1.13 (0.03)[c]	1.66 (0.04)	1.50 (0.04)	1.90 (0.03)
Petiole[b]	1.20 (0.05)	1.47 (0.04)	1.04 (0.03)	1.47 (0.05)
Pedicel[b]	0.88 (0.02)	1.15 (0.03)	0.82 (0.02)	1.98 (0.04)

[a] Hypocotyl length (in mm) of seedlings grown on MS medium with or without 5 μM GA_3. [b] The petiole and pedicel length (in cm) of plants sprayed twice a week with 50 μM GA_3 in 0.01% (w/v) Tween-20 (+GA) or with 0.01% (w/v) Tween-20 (−GA). [c] The nos. in parentheses denote the SE.

zinc-binding motifs of the $CX_9CX_{10}CX_2H$ type (Raventós et al., 1998).

Although the amino acids between the three Cys residues and the His residue in the $CX_9CX_{10}CX_2H$ motif of Os-GRF1 and HRT are not conserved, the spacing of the Cys and His residues is identical in both. In several classes of transcription factors, an NLS was found to overlap with or to be adjacent to a DNA-binding domain (Varagona and Raikhel, 1994; Hicks and Raikhel, 1995). Since the WRC domain may include both a NLS and a zinc finger, it is likely to function in DNA binding. The region that follows the WRC domain possesses sequences rich in Pro/Ser/Thr, His, and acidic amino acids, as well as a homopolymeric stretch of Gln. These too are features found in transcription factors and transcriptional activators (Mitchell and Tjian, 1989; Gerber et al., 1994). The third conserved motif of Os-GRF1, TQL, is the distinguishing characteristic of a subset of plant proteins with the QLQ and WRC domains. The TQL domain has not been recognized before in plant proteins, and its function is not yet known.

Os-GRF1 may therefore belong to a family of transcriptional regulatory proteins that are characterized by the QLQ and WRC motifs. The QLQ motif appears to be present in all eukaryotes. In contrast, the WRC motif appears to be unique to plants. The QLQ and WRC domains occur often in the same plant protein and may constitute a functional unit, the QLQ domain, which mediates homo- and/or heterodimerization between family members of this class of proteins and the WRC domain acting in DNA binding. Alternatively, the QLQ domain may be essential in the interaction with other proteins that are part of the transcriptional machinery.

Role of Os-GRF1 in Plant Growth and Development

RNA gel-blot analysis showed that *Os-GRF1* is specifically expressed in regions of the rice plant that contain meristematic tissues. In the rice internode, *Os-GRF1* transcripts were only detected in the intercalary meristem, which is the primary site of GA action (Sauter et al., 1993). The basal level of *Os-GRF1* mRNA was very low in the intercalary meristem but increased sharply after 2 h of GA treatment and 4 h of submergence. GA induces internodal elongation with a lag time of 40 min, and the initial growth response is due to increased cell elongation. Enhanced cell division activity commenced approximately 4 h after the start of treatment with GA (Sauter and Kende, 1992). The increase in the transcript level of *Os-GRF1* was apparent after cell elongation had started but before the increase in transcript levels of the cell-cycle-related genes encoding histone H3 and the mitotic cyclin cycOs1 was evident. The time course of GA-induced cell elongation and cell division activity in the intercalary meristem suggests that Os-GRF1 may be involved in mediating an early event in GA-promoted entry into the cell cycle.

To further investigate the role of Os-GRF1 in growth, we transformed Arabidopsis with *Os-GRF1*. Rather than promoting growth, expression of *Os-GRF1* in Arabidopsis led to severely reduced elongation of the primary inflorescence stem. This phenotype could have resulted from a gain-of-function with respect to one component of growth and, thereby, to an imbalance between growth-related processes. For example, expression of *Os-GRF1* in Arabidopsis may lead to excessive cell proliferation in the shoot apical meristem and, as a consequence, to the observed growth inhibition and fasciation (Van der Knaap, 1998). Alternatively, transformation of Arabidopsis with the rice gene may yield a dominant-negative phenotype. Os-GRF1 may displace the Arabidopsis GRF1 from a protein complex, thereby inactivating the latter.

The dwarf phenotype of Arabidopsis plants expressing *Os-GRF1* was not reversed by application of GA. Other growth processes, such as hypocotyl, petiole, and pedicel elongation, were promoted by GA to a similar extent as in control plants. Because stem growth was severely inhibited but other growth processes were not affected, it appears that expression of *Os-GRF1* in Arabidopsis disrupts functioning of the shoot apical meristem. GA not only plays a role in promoting stem, hypocotyl, and petiole elongation, but also in regulating germination, flowering, male fertility, and phase changes. Whereas stem elongation, germination, and male fertility were affected in Arabidopsis plants expressing *Os-GRF1*, other GA-controlled functions appeared to be normal. These observations indicate that GRF1 is not involved in regulating a basic reaction shared by all GA responses. Processes in which GA is not known to play a role, such as carpel and leaf development, were also affected by expression of *Os-GRF1* in Arabidopsis (van der Knaap, 1998). It is not known whether these pleiotropic effects reflect the actual role of GRF1 in plant development, or whether they are caused by ectopic expression of the heterologous gene in Arabidopsis and interference with signal transduction pathways that are regulated by GRF1-like proteins.

In conclusion, we have identified a new family of putative regulatory proteins in plants that are typified by the QLQ and WRC motifs. The prototype member of this family, Os-GRF1, is targeted to the nucleus, and several of its structural features indicate that it may play a role in controlling transcriptional activity. Both the expression pattern of *Os-GRF1* in rice and the phenotype of Arabidopsis plants transformed with *Os-GRF1* indicate that Os-GRF1 may be involved in mediating GA-dependent stem growth. Elucidating the function of Os-GRF1 will contribute to our understanding of GA action and the regulation of stem elongation.

ACKNOWLEDGMENTS

We thank Dr. Marguerite Varagona and Emily Avila-Teeguarden (then at New Mexico State University, Las Cruces) for performing the nuclear localization experiments; Cassandra Zylstra for technical help in making measurements of Arabidopsis plants; Drs. Anthony Bleecker (University of Wisconsin, Madison), Michael Thomashow, and Steven Triezenberg (both at Michigan State University, East Lansing) for critical reading of the manuscript; and the National Institute of Agrobiological Resources, Tsukuba, Japan, for providing the rice EST *D40170*.

Received November 10, 1999; accepted November 29, 1999.

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