Interaction between the GROWTH-REGULATING FACTOR and KNOTTED1-LIKE HOMEBOX Families of Transcription Factors


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KNOTTED1-LIKE HOMEBOX (KNOX) genes are important regulators of meristem function, and a complex network of transcription factors ensures tight control of their expression. Here, we show that members of the GROWTH-REGULATING FACTOR (GRF) family act as players in this network. A yeast (Saccharomyces cerevisiae) one-hybrid screen with the upstream sequence of the KNOX gene OsKno2 from rice (Oryza sativa) resulted in isolation of OsGRF3 and OsGRF10. Specific binding to a region in the untranslated leader sequence of OsKno2 was confirmed by yeast and in vitro binding assays. ProOskn2β-glucuronidase reporter expression was down-regulated by OsGRF3 and OsGRF10 in vivo, suggesting that these proteins function as transcriptional repressors. Likewise, we found that the GRF protein BGRF1 from barley (Hordeum vulgare) could act as a repressor on an intron sequence in the KNOX gene Hooded /Barley Knootted 3 (Bkn3) and that AtGRF4, AtGRF5, and AtGRF6 from Arabidopsis (Arabidopsis thaliana) could repress KNOTTED-LIKE FROM ARABIDOPSIS THALIANA2 (KNAT2) promoter activity. OsGRF overexpression phenotypes in rice were consistent with aberrant meristematic activity, showing reduced formation of tillers and internodes and extensive adventitious root/shoot formation on nodes. These effects were associated with down-regulation of endogenous OsKno2 expression by OsGRF3. Conversely, RNA interference silencing of OsGRF3, OsGRF4, and OsGRF5 resulted in dwarfism, delayed growth and inflorescence formation, and up-regulation of OsKno2. These data demonstrate conserved interactions between the GRF and KNOX families of transcription factors in both monocot and dicot plants.

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6 This article is dedicated to the memory of Rolf J. de Kam, who passed away during the period of this study.

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KNOTTED1-LIKE HOMEBOX (KNOX) class I homeobox genes play an essential role in the development and maintenance of the shoot apical and floral meristems (Hake et al., 1995, 2004; Endrizzi et al., 1996; Hake, 1996; Reiser et al., 2000; Brand et al., 2002; Hake and Ori, 2002; Ito et al., 2002; Tsuda et al., 2011). KNOX proteins contribute to the regulation of meristem maintenance by regulating the production of GAs. Different KNOX genes have been shown to inhibit GA biosynthesis in the shoot apical meristem (SAM) through down-regulation of the key biosynthetic gene GA20 oxidase (Kusaba et al., 1998; Tanaka-Ueguchi et al., 1998; Sakamoto et al., 2001; Hay et al., 2002; Rosin et al., 2003) or by controlling the level of GA2 oxidase1 that degrades GA (Balduc and Hake, 2009). Down-regulation of KNOX gene expression at the flanks of the SAM is thought to permit biosynthesis of GA and consequently to result in organized cell proliferation and determination of cell fate (Sakamoto et al., 2001).

A large body of evidence suggests that the precise regulation of KNOX activity is central to the determination of organ versus meristem identity in a wide range of plant species (Hake et al., 1995, 2004; Hake, 1996; Reiser et al., 2000). In the monocot plants rice (Oryza sativa) and...
maize (*Zea mays*), misexpression of *KNOX* genes has a profound effect on the blade-sheath boundary in the leaves (Friefeld and Hake, 1985). In Arabidopsis (*Arabidopsis thaliana*), *KNOX* misexpression also affects leaf formation, leading to serrations and the formation of ectopic meristems in the sinuses (Chuck et al., 1996; Long et al., 1996; Dean et al., 2004; Kuijt et al., 2004). A loss-of-function mutation of BREVIPEDELICUS (BP) in Arabidopsis causes defects in stem elongation due to a lower number of cell divisions and defects in the differentiation and elongation of epidermal and cortical cells (Venglat et al., 2002). The phenotypes of shoot meristemless (*stm*) alleles range from a reduced SAM size to a complete lack of the meristem and fused cotyledons (Endrizzi et al., 1996; Venglat et al., 2002).

*KNOX* gene expression is regulated at multiple levels to prevent misexpression in leaves and leaf primordia (Hibara et al., 2002; Kumaran et al., 2002; Kim et al., 2003b; Lin et al., 2003; Kuijt et al., 2004). Several negative regulators of *KNOX* gene expression have been identified because their loss-of-function phenotypes resemble the phenotypes of *KNOX* overexpressors. MYB domain transcription factors are such negative regulators conserved between maize (rough sheath2), *Antirrhinum majus* (PHANTASTICA), and Arabidopsis (ASYMMETRIC LEAVES1 [AS1]; Waite et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000, 2002). In Arabidopsis, a network of negative interactors provides a mechanism to distinguish between founder cells and stem cells in the SAM. *AS1* plays a central role in this network, repressing the activity of *BP* and KNOTTED-LIKE FROM *ARABIDOPSIS THALIANA*2 (KNAT2) and being negatively regulated itself by *STM* (Byrne et al., 2002). *AS1* forms a complex with *AS2*, a member of the LATERAL ORGAN BOUNDARY DOMAIN (LBD) family, to create a hairpin in the promoters of *BP* and KNAT2 that blocks their transcription (Guo et al., 2008). Other negative regulators of *KNOX* gene expression include members of BLADE ON PETIOLE, YABBY, and BEL1-like gene families (Kumaran et al., 2002; Ha et al., 2003; Kumar et al., 2007). Positive regulators of *KNOX* gene expression have also been described, such as genes of the CUP-SHAPED COTYLEDON (CUC) and the LBD gene families, with members CUC1, CUC2, and JAGGED LATERAL ORGANS (JLO) genes (Hibara et al., 2002; Borghi et al., 2007). The expression patterns of CUC1, CUC2, and STM overlap during early embryogenesis in the region of the organizing SAM (Aida et al., 1999; Takada et al., 2001). Overexpression of CUC1 leads to ectopic expression of *STM*, *BP*, KNAT2, and KNAT6 in cotyledons (Hibara et al., 2002). The microRNA miR164A regulates the extent of serrations by regulating the expression of CUC2 in the leaf sinuses (Nikovics et al., 2006). Misexpression of *JLO* increases the expression of *STM* and KNAT1 in leaves, whereas the *ifo-D* mutant has small lobed leaves resembling the *bp* mutant phenotype (Borghi et al., 2007).

Previously, we addressed the transcriptional control of a rice *KNOX* gene, *Oskn2*, showing that its promoter sequence is sufficient to mediate the initial down-regulation in developing organ primordia (Postma-Haarasma et al., 2002). Here, we used the *Oskn2* promoter as a bait sequence in a yeast (*Saccharomyces cerevisiae*) one-hybrid screen aimed at the identification of novel upstream regulators. This screen resulted in the isolation of two members of the GROWTH-REGULATING FACTOR (GRF) family, OsGRF3 and OsGRF10, both interacting specifically with the *Oskn2* promoter region. The first member of the rice *GRF* family, named *OsGRF1*, was described by van der Knaap et al. (2000) and isolated in a search for genes that are differentially expressed in the intercalary meristem of deepwater rice internodes in response to GA. Two highly conserved regions, the QLQ and WRC domains, are distinctive characteristics of the *GRF* family members in both monocot and dicot species (van der Knaap et al., 2000; Kim et al., 2003a; Choi et al., 2004; Zhang et al., 2008; Osnato et al., 2010). In this report, we provide evidence of functional links between *GRF* proteins and transcriptional regulation of *KNOX* genes. We show that OsGRF3 and OsGRF10 repress *Oskn2* promoter activity in planta. Likewise, a GRF protein from barley (*Hordeum vulgare*) named BGRF1 (Osnato et al., 2010) was found to act as a repressor on an intron sequence in the barley KNOX gene *Hooded*/*Bkn3*, and three Arabidopsis GRF proteins could repress KNAT2 promoter activity. These data support the proposition that repressor activity on *KNOX* genes is a conserved function of members of the *GRF* family in monocot and dicot plant species.

**RESULTS**

**Specific Interaction of OsGRF3 and OsGRF10 Proteins with the Oskn2 Promoter in Yeast**

To identify novel genes involved in the regulation of the rice KNOX class I gene *Oskn2*, we performed a yeast one-hybrid screen. A rice zygote-derived embryonic complementary DNA (cDNA) expression library in pACTII vector was used as prey, and HIS3 reporter gene constructs containing promoter fragments of 662 and 1,264 bp upstream of the ATG of *Oskn2* were used as bait. In three independent rounds, we screened a total of 900,000 yeast transformants with the short bait construct (ProOskn2S:HIS3) and 700,000 transformants with the long bait (ProOskn2L:HIS3). This resulted in 18 positive clones, which, after sequence analysis, all showed homology to the rice gene encoding OsGRF1, the founding member of the *GRF* family of putative transcription factors (van der Knaap et al., 2000). The clones obtained represented two genes, designated OsGRF3 (two clones with the short bait) and OsGRF10 (14 clones with the short bait and two clones with the long bait). By comparison with an OsGRF3 EST sequence (GenBank accession no. AU182732) and TIGR gene model 12004m.10015, we derived that both OsGRF3 clones from our screen encoded a partial OsGRF3 sequence lacking 15 amino acids at the N-terminal end. This partial sequence was in frame with the Gal4p activation domain (AD) sequence in the pACTII vector. To obtain a full-length OsGRF3 clone, we rescreened the library using the short bait construct and performed colony PCR on positive yeast transformants.
This resulted in six clones encoding the full-length OsGRF3 sequence of 384 amino acids in frame with the Gal4 AD. The OsGRF10 clones from our screen were also in frame with the Gal4 AD and corresponded to two different splice variants of the OsGRF10 gene, one fitting with TIGR gene model 12002m.33821 (nine clones) and the other fitting with gene model 12002m.09594 (five clones). The two OsGRF10 splice variants are 209 and 211 amino acids long and differ only at the C-terminal end. The cDNA encoding the 211 amino acid variant was used in this study.

The OsGRF3 and OsGRF10 clones in pACTII were retransformed into the ProOskn2::HIS3 reporter strains and were shown in a titration experiment to grow on media containing up to 25 mM of 3-aminotriazol (3-AT); a competitive inhibitor of the His-3p reporter protein, thereby confirming activation of the ProOskn2::HIS3 reporter constructs (data not shown). To determine the DNA-binding specificity of the OsGRF3 and OsGRF10 factors, we used yeast strains containing HIS3 reporter gene constructs with different fragments of the 5′ regulatory sequences of two other rice KNOX class I genes, Oskn1 and Oskn3. As shown in Supplemental Figure S1 and summarized in Table I, factors OsGRF3 and OsGRF10 were not able to activate these other reporter constructs, indicating that their interaction with the Oskn2 promoter is specific.

Conserved Binding of Rice and Arabidopsis GRF Proteins to the Oskn2 Promoter

To map the region where OsGRF3 and OsGRF10 proteins interact with the Oskn2 promoter, we divided the 662-bp promoter fragment used in the screen into three subfragments (A, B, and C) of 230 bp, with overlaps of 10 and 16 bp (Fig. 1A). Reporter constructs of these subfragments were integrated into the yeast genome. OsGRF3 and OsGRF10 could not activate the reporter with subfragment A but were able to activate both the reporters with fragments B and C (Table II; Supplemental Fig. S2). Based on this result, a fourth reporter strain was constructed, containing a 34-bp promoter fragment (D) overlapping with the distal end of subfragment B and the proximal end of subfragment C (Fig. 1A). Both OsGRF3 and OsGRF10 were also able to activate the reporter construct with subfragment D (Table II; Supplemental Fig. S2).

To investigate whether subfragment D contains a conserved GRF binding site, we tested three distantly related GRF proteins from Arabidopsis, AtGRF4, AtGRF5, and AtGRF6 (Kim et al., 2003a). Like OsGRF3 and OsGRF10, all three Arabidopsis GRF proteins in vector pACTII could activate the reporter with subfragment D, while none of them activated the reporter with subfragment A (Table III; Supplemental Fig. S3). AtGRF4, AtGRF5, and AtGRF6 were also able to activate the reporter with subfragment B, but only AtGRF5 could additionally activate the reporter construct with subfragment C (Table III; Supplemental Fig. S3).

Binding of OsGRF3 and OsGRF10 proteins to subfragments of the Oskn2 promoter was confirmed in vitro using glutathione S-transferase (GST) fusion proteins in electrophoretic mobility shift assays (EMSAs; Fig. 1). A DNA-protein complex was observed for the subfragments B, C, and D, but not for subfragment A (Fig. 1, A and B), fully consistent with the results obtained in yeast. GST fusion proteins of Arabidopsis AtGRF4, AtGRF5, and AtGRF6 were also able to bind to subfragment D, whereas GST control protein was not. Based on the combined results of yeast one-hybrid and EMSA analysis, we conclude that specificity for a DNA-binding site within the 34-bp sequence from Oskn2 promoter subfragment D is conserved at least among the five tested members of the GRF family in monocot and dicot plant species. Subfragments B, C, and D are located between positions –538 and –83 upstream of where we assume the start codon of Oskn2 is located (as indicated in GenBank accession no. AF323785).

Based on comparison of the genomic sequence of Oskn2 (UniGene Os.4162) with 41 EST clones (of which GenBank accession no. CI740419 is the longest at the 5′ end), we define the putative mRNA transcription start at position –605. Therefore, OsGRF3 and OsGRF10 appear to interact with the 5′-untranslated region (UTR) of Oskn2.

Furthermore, analysis of deletion constructs of OsGRF10 showed that binding to subfragment D requires both the conserved QLQ and WRC domains described by van der Knaap et al. (2000; Table IV; Supplemental Fig. S2C). The QLQ domain was proposed to be involved in protein-protein interactions based on homology with the Switch/Sucrose Nonfermentable (SWI2/SNF2) chromatin remodeling complex in yeast (van der Knaap et al., 2000). The WRC domain contains a nuclear localization signal and a CCCH (C3H)-type zinc-finger motif, consistent with a function in DNA binding and transcriptional control (van der Knaap et al., 2000). Our results are in line with results of Osnato et al. (2010), who showed that deletion of the C3H zinc-finger motif of the WRC domain abolished DNA binding in vitro. However, this does not exclude the possibility that other domains of GRF factors also contribute to DNA binding.

Table I. Yeast one-hybrid assays with OsGRF3 and OsGRF10 expression constructs and KNOX promoter HIS3 constructs

<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>pACTII-OsGRF3</th>
<th>pACTII-OsGRF10</th>
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<tr>
<td></td>
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<tr>
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<td>YPO101</td>
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Recently, Kim et al. (2012) identified a binding site for Arabidopsis GRF7 with the core sequence TGTCAGG. We checked the Oskn2 promoter for homology and found that the subfragments binding OsGRF3 and OsGRF10 (B, C, and D) are enriched for the motif CAG, which is contained within this core sequence. Subfragment D contains a cluster of seven CAG repeats or of its reverse complementary sequence CTG. Subfragment B has 14 CAG or CTG repeats (without the overlapping subfragment D), of which most are clustered in three regions with three or four repeats. Subfragment C also has 14 CAG or CTG repeats (without the overlapping subfragment D), of which one-half are in one cluster. By contrast, subfragment A, which has the same length as B and C, did not show any GRF binding in the yeast assays or the EMSAs and has only three CAG or CTG repeats, which are not clustered. Furthermore, the 305-bp fragment from the Bkn3 intron that is bound by BGRF1 has 14 CAG or CTG repeats, including one cluster of four repeats and two clusters of two repeats (Osnato et al., 2010). Taken together, the data suggest that GRF binding is associated with the presence of CAG or CTG repeats.

Expression Patterns of OsGRF3 and OsGRF10 Are Partially Overlapping with Oskn2

As shown above, yeast one-hybrid and in vitro binding studies have demonstrated DNA binding of GRF proteins to the Oskn2 promoter. Consistent with a possible transcription factor function, GFP tagging resulted in nuclear localization upon transient expression in onion (Allium cepa) cells (Supplemental Fig. S4A). Next, we compared expression patterns of the GRF genes in relation to their predicted target gene, Oskn2. To this end, we analyzed mRNA samples from 13 different tissues by reverse transcription (RT)-PCR (Fig. 2). OsGRF3 was broadly expressed in all tissues tested, except in roots. By contrast, OsGRF10 was only expressed in embryos, seedlings, immature rachis, and mature nodes but not in other tissues from mature plants, which is also confirmed.

Table II. Interaction of OsGRF3 and OsGRF10 proteins with Oskn2 promoter subfragments A, B, C, and D

<table>
<thead>
<tr>
<th>Yeast Strain</th>
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<th>pACTII-OsGRF10</th>
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<tr>
<td>ProOskn2-D</td>
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Figure 1. In vitro interactions of GST-OsGRF and GST-AtGRF proteins with Oskn2 promoter fragments in EMSAs. A, Subfragments A, B, and C of the Oskn2 promoter were tested for interaction with GST, GST-OsGRF3, and GST-OsGRF10 proteins. Bottom, Schematic organization of the Oskn2 promoter in subfragments A to D. B, Complex formation of GST-AtGRF4, GST-AtGRF5, GST-AtGRF6, GST-OsGRF3, and GST-OsGRF10 proteins with Oskn2 promoter fragment D. Similar to the yeast assays (Supplemental Figs. S1–S3), the GRF proteins interacted with Oskn2 promoter subfragments B, C, and D. GST was used as a control protein and shows no binding.

Table II. Interaction of OsGRF3 and OsGRF10 proteins with Oskn2 promoter subfragments A, B, C, and D

Constructs pACTII-OsGRF3, pACTII-OsGRF10, and pACTIIa were analyzed in yeast strains Y187::ProOskn2-A, Y187::ProOskn2-B, Y187::ProOskn2-C, and Y187::ProOskn2-D on medium containing His or supplemented with 10 mM 3-AT (and without His). The locations of the Oskn2 promoter subfragments are schematically indicated in Figure 1A. Results are based on Supplemental Figure S2.
by promoter GUS analysis (Supplemental Fig. S4). In several tissues, both Os kn2 and the GRF genes were found to be expressed, but Os kn2 was also expressed in roots where the GRF genes were not expressed. On the other hand, Os kn2 expression was absent in the region just above the SAM, which consist of leaf sheath and young leaves rolled upon each other, where OsGRF3 and OsGRF10 were both expressed.

**Ectopic Expression of OsGRF3 and OsGRF10 in Rice Reduces TILLERING and Induces Ectopic Root and Shoot Formation on Nodes**

To study the possible function of the GRF factors in regulation of KNOX genes, we generated rice plants in which the Cauliflower mosaic virus (CaMV) 3SS promoter drives overexpression of the full-length cDNAs of OsGRF3 (two independent lines) and OsGRF10 (seven independent lines) or a partial OsGRF3 cDNA (OsGRF3s) from the original yeast screen, expressing a shorter protein from an internal ATG (five independent lines). Overexpression lines (T1 generation) of all three constructs showed similar phenotypical alterations, although effects of the longer OsGRF3 cDNA were stronger than effects of the shorter version and the effects of OsGRF10. The T1 generation of OsGRF3s, OsGRF3, and OsGRF10 overexpressors segregated into three classes, showing severe, mild, or no phenotypical changes. PCR analysis confirmed the presence of the transgene in the plants with mild and severe phenotypes and its absence in plants without phenotypical changes (data not shown). Normally, the stem of a rice plant produces a large number of tillers, reaching around 50 or even more at the maximum tiller stage under field conditions. The tillers arising on a main stem are called primary tillers. Secondary tillers can develop from the axis of leaves. T1 progeny of all independent overexpression lines contained plants with a mild phenotype, forming three to four tillers (n = 16, 3.4 ± 0.8) in the time that wild-type plants produced four to six (n = 14, 5.1 ± 1.1). Furthermore, they formed adventitious roots on the nodes of tillers (Fig. 3C), which was not observed in the wild type (Fig. 3B). The severe class of GRF overexpressors (T1 progeny of two OsGRF3 lines, four OsGRF3s lines, and two OsGRF10 lines) consisted of slender plants with only one or two long tillers (Fig. 3, A and E), which had swollen nodes, showing extensive formation of adventitious roots and bundles of ectopic shoots (Fig. 3D). Increased tiller length was due to an increase in internode number, whereas internode lengths were not significantly increased (Supplemental Fig. S5). Inflorescence formation of the overexpressors was delayed approximately 2 weeks (OsGRF3s and OsGRF10 lines) up to 3 months (OsGRF3 lines), and the most severely affected plants (OsGRF3 lines) senesced when inflorescences were still enclosed within the leaf sheath. A region with a distinctive epidermal structure was noticed at the base of leaf blades of the overexpressors. This phenotype, affecting only the epidermis but not the internal leaf structure, was most apparent in OsGRF3 overexpressors. Scanning electron microscopy (SEM) analysis showed that the epidermis had formed fewer specialized epidermal structures such as silica knobs, bristles, and macro hairs and that the distance between stomata was decreased (Fig. 3, F and G). This phenotype was distinct from that of Os kn2 overexpressors or other rice KNOX gene overexpressors in which sectors

<table>
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<th>Yeast Strain</th>
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Table IV. Analysis of DNA-binding properties of the QLQ and WRC domains from OsGRF10 in yeast one-hybrid screens

Yeast strains Y187::ProOskn2-662 and Y187::ProOskn2-D harboring pACTII-QLQ, pACTII-WRC4, pACTII-QLQ-WRC4, or pACTII-QLQ-WRC5 were examined for growth on medium containing His or medium supplemented with 10 mM 3-AT (without His). Individual QLQ and WRC domains cannot activate the ProOskn2-HIS3 reporters when fused to the Gal4p AD in pACTII, whereas the combination QLQ-WRC can. Results are based on Supplemental Figure S2C.
showing sheath-like epidermal and internal leaf organization were displaced over the leaf blade area (Matsuoka et al., 1993; Postma-Haarsma et al., 2002). In conclusion, OsGRF3 and OsGRF10 overexpression inhibited the formation of new tillers, while increasing the formation of nodes and adventitious roots and shoots within the primary tiller(s). Additionally, OsGRF3 and OsGRF10 overexpression affected the epidermal structure in basal sectors of the leaf blade.

KNOX Gene Expression Is Down-Regulated by GRF Overexpression

The expression of KNOX genes was analyzed in T2 seedlings of OsGRF3 overexpressors. This analysis could not be performed for OsGRF10 overexpressors because the T1 generation did not produce seeds. Down-regulation of Oskn1, Oskn2, and Oskn3 expression was observed by quantitative PCR (qPCR) analysis on RNA samples from SAM-containing (bottom) stem segments of OsGRF3 overexpressing seedlings (Fig. 4). Taken together with our DNA binding studies, these data strongly suggest that OsGRF3 acts as transcriptional repressor of Oskn2 and that Oskn1 and Oskn3 may be down-regulated by an indirect mechanism, because GRF proteins only showed a direct interaction with the Oskn2 promoter.

KNOX Gene Expression Is Up-Regulated by RNA Interference (RNAi)-Mediated OsGRF Silencing

Because no suitable transfer DNA (T-DNA) or transposon insertion mutants for OsGRF3 or OsGRF10 were available, an RNAi approach was set up. For this, a construct was used based on the WRC motif from a barley GRF gene (BGRF1) that shares 93%, 85%, and 73% identity, respectively, with OsGRF3, OsGRF4, and OsGRF5, which are, according to a phylogeny reconstruction (Supplemental Figs. S6–S9), all in subclass C (Zhang et al., 2008). Different T0 lines exhibiting OsGRF gene silencing were selected (lines 1.2 and 1.4), self-pollinated, and amplified to T1 and T2 generations. T2 RNAi rice plants were analyzed by qPCR to assess the expression levels of endogenous GRF genes. As shown in Figure 5A, the expression of OsGRF3 and OsGRF4 was greatly reduced in the lines examined, whereas the expression of OsGRF5 was less affected likely due to the lower sequence similarity with the sequence of BGRF1 (Supplemental Figs. S6 and S8). Silencing of rice GRF genes caused developmental alterations, including...
reduced plant height (Fig. 5, C and D), which were strongest in line 1.2. The average plant height of the cv Nipponbare controls was 54.1 cm, which dropped to 28.1 and 44.2 cm for lines 1.2 and 1.4 (Fig. 5E), respectively. This phenomenon is largely caused by reduced internode length, because the average internode number (on the master tiller) stayed with 3.2, the same in line 1.4, and dropped only slightly to 2.4 in line 1.2 (Fig. 5F), but is not statistically significant. A strong effect was found on tiller number, which dropped from an average of 12.8 for the wild type to 1.3 and 5.4 for lines 1.2 and 1.4 (Fig. 5G), respectively. These phenotypic effects were accompanied by up-regulation of Oskn1, Oskn2, and Oskn3 gene expression, as revealed by qPCR analyses on a seedling sample corresponding to a 1-cm region of leaf blade and sheath comprising the SAM (Fig. 5B). These data are consistent with the GRF overexpression analysis, which resulted in down-regulation of the same KNOX genes.

OsGRF3 and OsGRF10 Repress ProOskn2:GUS Expression in Rice Calli

To provide further evidence for the regulatory function of the GRF proteins, we set up a transient expression system based on agroinoculation with Agrobacterium tumefaciens strains transferring T-DNA constructs expressing either OsGRF3 or OsGRF10. These T-DNA constructs were transferred to embryonic rice calli harboring construct ProOskn2:GUS. In this construct, the GUS gene is driven by the promoter of the Oskn2 gene, which was described before (Postma-Haarsma et al., 2002). Calli at 2, 4, 6, 8, and 10 d post inoculation (dpi) with A. tumefaciens were stained with X-Gluc, and ProOskn2:GUS expression was quantified from stereomicroscope images using ImageJ. Agroinoculation with both OsGRF3 and OsGRF10 constructs resulted in a decrease of the Oskn2 promoter-driven GUS expression at 4, 6, 8, and 10 dpi compared with the expression at 2 dpi and compared with control calli that were treated with an A. tumefaciens strain transferring construct Pro-35S:GFP (Supplemental Fig. S10, A and B). These results demonstrate that when OsGRF3 and OsGRF10 are overexpressed in ProOskn2:GUS calli, Oskn2 promoter-driven GUS expression decreases, indicating that both GRF proteins act as repressors in this experimental set up.

GRF-KNOX Interactions Are Conserved in Barley and Arabidopsis

In a yeast one-hybrid approach, Osnato et al. (2010) cloned BGRF1, a member of the GRF family, from barley and one of several transcription factors interacting with a 305-bp sequence from intron IV of the barley KNOX class I gene Bkn3. A duplication of this sequence causes ectopic expression of Bkn3 that leads to the so-called Hooded phenotype (Müller et al., 1995). Here, we used rice protoplast assays to investigate the interaction of BGRF1 with the intron IV 305-bp element. It has been shown that expression of a GUS reporter construct consisting of the 305-bp element upstream of a minimal CaMV 35S promoter can be activated by transcription factor Barley Ethylene Insensitive-Like1 (BEIL1) (Osnato et al., 2010). We found that cotransformation of BGRF1 resulted in repression of BEIL1-activated expression of the reporter construct (Fig. 6). This result supports the ability of both BGRF1 and BEIL1 to bind in planta to the 305-bp element and is consistent with EMSA experiments that showed that BGRF1 binds distal to the 305-bp sequence, whereas BEIL1 binds in the center (Osnato et al., 2010). OsGRF10 could substitute for BGRF1 as a repressor of BEIL1-activated reporter gene expression (Fig. 6), indicating species-conserved binding specificity and repressor activity of the GRF proteins.

To obtain additional experimental support for the interaction between GRF proteins and KNOX genes, we used GUS reporter lines of Arabidopsis driven by
promoter sequences of the KNOX class I genes BP/KNAT1 (Öri et al., 2000; ProBP:GUS) and KNAT2 (Dockx et al., 1995; ProKNAT2:GUS) and stably transformed these with overexpression constructs of OsGRF10 and AtGRF4, AtGRF5, and AtGRF6. ProKNAT2:GUS expression was concentrated in the SAM of seedlings and in part of the hypocotyl but absent from the petioles of leaves (Supplemental Fig. S11E). By contrast, seedlings overexpressing OsGRF10, AtGRF4, AtGRF5, or AtGRF6 showed developmental aberrations (Supplemental Fig. S11, L–O and Q–T), and the expression of ProKNAT2:GUS was reduced to a weak spot around the SAM (Supplemental Fig. S11, A–D). Thus, the same rice and Arabidopsis GRF proteins that showed conserved binding to a 34-bp subfragment of the Oskn2 promoter were able to down-regulate ProKNAT2:GUS expression. By contrast, ProBP:GUS expression was not affected by overexpression of any of these four GRF

Figure 5. Down-regulation of GRF genes in rice results in up-regulation of KNOX genes and affects plant architecture. A and B, Relative expression levels of endogenous OsGRF3, OsGRF4, and OsGRF5 (A) and Oskn1, Oskn2, and Oskn3 genes (B) in BGRF1 RNAi lines 1.2 and 1.4 (T2 generation), respectively. Total RNA was extracted from a pool of leaves of five 10-d-old seedlings. The OsUbi gene was used to normalize data, and cv Nipponbare was used as wild-type control. qPCR was performed in triplicate, and mean values with s are shown. Two independent biological replicates were performed with similar results. The data were analyzed with an unpaired Student’s t test. Asterisks indicate significant differences (P < 0.05) compared with the untransformed controls. C and D, Phenotypes of greenhouse-grown rice plants at anthesis stage expressing the BGRF1 RNAi construct. Line 1.2 (C) has a stronger phenotype than line 1.4 (D). The control cv Nipponbare plants are at the left and right, and four T2 plants are in the middle. E to G, The BGRF1 RNAi plants were phenotyped for plant height (E), internode number on the master tiller (F), and tiller number (G). The mean values with s of two lines (n = 15 and n = 29 plants for lines 1.2 and 1.4, respectively) are compared with wild-type cv Nipponbare (NB; n = 5), and the data were analyzed using ANOVA followed by Bonferroni corrections. Asterisks indicate significant differences (P < 0.05) compared with the untransformed controls. 
Figure 6. Interaction of BGRF1 and OsGRF10 with the 305-bp Bkn3 intron IV element in rice protoplasts. Effects of BGRF1 and OsGRF10 overexpression on down-regulation of BEIL1-activated GUS reporter gene expression were tested using reporter constructs K373 and K373 +305 bp. Construct K373 harbors the GUS gene preceded by the –46 minimal promoter derived from ProCaMV 35S. Construct K373+305 bp contains the 305-bp enhancer sequence from intron IV of Bkn3 upstream of the minimal CaMV 35S promoter. GUS activities were normalized on FLUC activity, which served as a control for transformation efficiency. Bars in the histogram indicate GUS/FLUC activity ratio of effector with K373+305 bp with respect to the value obtained with the same effector and the control reporter K373. The graph reports mean values of six independent transformations of each construct combination, and error bars represent SD values. BEIL1 activates construct K373+305 bp with a factor 4.2, which is lowered to 3.1 and 2.6 in the presence of BGRF1 and OsGRF10, respectively. Six individual transformations per construct were used in each of the measurements, and the data were analyzed using ANOVA followed by Bonferroni corrections. Letters have been assigned to distinct groups based on 95% confidence intervals.

proteins (Supplemental Figure S11, F–J), indicating that the interaction with the KNAT2 promoter is specific. As discussed above, GRF binding activity to Oskn2 promoter fragments was associated with the presence of CTG or CAG repeats. We checked Bp and KNAT2 promoter regions of 1 kb upstream of the ATG start codon and found 16 CTG and CAG repeats in the KNAT2 promoter, whereas the Bp promoter, which was not repressed by GRFs, only contained three such sequences.

DISCUSSION

Here, we report on two factors of the GRF family, OsGRF3 and OsGRF10, interacting with the 5' upstream sequence of the rice KNOX class I homeobox gene Oskn2. We found that these GRF proteins and homologs in barley and Arabidopsis act as transcriptional repressors on KNOX genes. KNOX expression is linked to SAM maintenance and organ development through tight control by a network of transcription factors (Byrne et al., 2000; Semiarti et al., 2001; Lin et al., 2003; Uchida et al., 2010; Tsuda et al., 2011). Most regulators of KNOX genes were identified through analysis of mutants resembling plants with altered KNOX expression. In this study, yeast one-hybrid screening revealed a novel interaction between OsGRF3 and OsGRF10 and Oskn2. We mapped binding of these GRFs to three promoter fragments of the 5' UTR region of Oskn2, suggesting that the interaction might prevent transcription. A similar regulatory mechanism was found for the murine Tumor Necrosis Factor-α gene, where mutation of a heat shock factor (HSF1) binding site in the 5' UTR abolishes HSF1-mediated repression (Singh et al., 2002).

Little is known about the precise functions of GRFs. Previously, rice GRF genes were shown to be regulated by GA1 (van der Knaap et al., 2000; Choi et al., 2004). Several KNOX genes from rice, tobacco (Nicotiana tabacum), and Arabidopsis have been implicated in regulation of GA biosynthesis by repression of a Gibberellic Acid-20 oxidase (GA20ox) gene (Kusaba et al., 1998; Sakamoto et al., 2001; Hay et al., 2002). However, we examined expression of OsGa20ox1 and OsGa20ox2 in OsGRF3 overexpressors as well as RNAi plants but did not find a significant difference (data not shown) that could explain the phenotypes we observed. Mutant analysis is hampered by the fact that single mutants for GRF1, GRF2, and GRF3 in Arabidopsis do not show many morphological changes, which may be due to redundancy, as expression patterns of different GRF genes are overlapping (Kim et al., 2003a; Horiguchi et al., 2005). Double and triple grf mutants for GRF1, GRF2, and GRF3 in Arabidopsis showed smaller leaf sizes, which could be attributed to reduced cell proliferation (Kim and Kende, 2004; Kim and Lee, 2006). Addition of a grf4 mutation to the grf1/grf2/grf3 triple mutant resulted in spoon- and cup-shaped cotyledons, and a significant portion of the mutant population showed the phenotype of the Arabidopsis stmnt mutant lacking a SAM (Chuck et al., 1996; Kim and Lee, 2006). Single mutants of grf4 or grf5 had smaller leaves, and GRF5 overexpression increased leaf size, suggesting a role in controlling cell number in leaves as also observed for GRF1 and GRF2 overexpression (Horiguchi et al., 2005). Recently, a function for GRF7 from Arabidopsis was described in repression of abscisic acid and osmotic stress-responsive genes including the transcription factor Dehydration-Responsive Element Binding2A (DREB2A) protein (Kim et al., 2012). Horiguchi et al. (2005) showed that all nine Arabidopsis GRF promoter constructs are highly expressed in leaf primordia and have different expression patterns that partially overlap with each other. Formation of leaf primordia is dependent on down-regulation of KNOX genes in the SAM (Byrne et al., 2000; Semiarti et al., 2001; Lin et al., 2003). Therefore, it is possible that GRFs may contribute to the down-regulation of KNOX activity during leaf primordium development in Arabidopsis, together with other transcriptional repressors for which such function has previously been demonstrated (Byrne et al., 2000; Semiarti et al., 2001; Lin et al., 2003). In rice, it is more difficult to speculate about the possible function of GRFs in relation to KNOX activity, as expression of both OsGRF10 and Oskn2 was absent from the seedling.
SAM and leaf primordia. However, at the late stage of embryo development, ProOsK2:GUS expression was down-regulated in the SAM when ProOsGRF10::GUS was still highly expressed, suggesting the possibility that OsGRF10 might repress OsK2 at this developmental stage. Other functional relationships between OsGRF10 and OsK2 might also exist, as their expression patterns were partially overlapping in several other tissues, including the vascular system.

OsGRF3 and OsGRF10 overexpression resulted in taller, slender plants, with reduced tillering, increased internode number, extensive adventitious roots and ectopic shoots on the nodes, delayed flowering, and abnormalities in leaf epidermis development. Only OsGRF3 overexpressors were fertile, and their seedlings showed OsK2 down-regulation. By contrast, RNAi silencing of OsK2 on the nodes, delayed regression. The effects on OsK1 and OsK3 expression might be indirect, because their respective promoters were not recognized by OsGRFs in yeast assays. Alternatively, it is possible that GRF binding sites might be located in the introns or further downstream of the open reading frame, as in the case of Bkb3 (Osnato et al., 2010). Such a direct interaction of GRFs with OsK1 and OsK3 is conceivable, because their respective expression patterns are overlapping in the SAM of developing embryos and in developing spikelets and leaf blades and sheaths (Sato et al., 1998; Postma-Haarsma et al., 1999, 2002; Sentoku et al., 1999). On the other hand, an indirect mechanism is also conceivable. Tsuda et al. (2011) showed, using ORYZA SATIVA HOMEobox1 (OSH1)/OsK1 overexpressors and an osh1osh15 double mutant together with in vivo binding assays, that this gene positively regulates at least four other KNOX I genes, including OSH71/OsK2 and OSH15/OsK3. This regulation occurs very likely through binding to conserved TGAC sequences. Similar results were obtained with maize KNOTTED1, which was shown among other KNOX genes to positively regulate liguleslessa (lg4a), which is orthologous to OsK2 (Bolduc et al., 2012). It is plausible that OsK2 has the same effect on expression of other KNOX class I genes in rice, because yeast assays have shown that OsK2 recognizes the same conserved TGAC sequences (Postma-Haarsma et al., 2002).

Further evidence for a repressor function of members of the GRF family was obtained from transient expression experiments with the BGRF1 gene from barley and analysis with Arabidopsis GRF overexpressing lines in a ProKNA2:GUS background. Similar as in rice, overexpression of GRFs in Arabidopsis did not result in typical KNOX overexpression phenotypes (Kuijt et al., 2004) but in plants showing severe defects in SAM and leaf development. This cannot be solely due to repression of KNA2 because knat2 does not show any obvious phenotype despite the predominant expression of KNA2 in the SAM (Byrne et al., 2002; Belles-Boix et al., 2006). Likely, the expression of other target genes involved in SAM development is also altered by GRF overexpression. Although we did not observe down-regulation of ProBP::GUS, we cannot exclude the possibility that the expression of the endogenous KNOX genes might be altered due to the presence of regulatory elements elsewhere in the gene as with barley Hooded (Müller et al., 1995; Osnato et al., 2010). In the rice OsGRF overexpressors, we did not observe similar SAM defects; however, the reduced tillering and adventitious root and shoot formation on the nodes are indications of aberrant meristematic activity. Further investigations will be required into the functional relationships between GRF and KNOX genes and biological processes involved. Furthermore, with the GRF7–DREB interaction confirmed (Kim et al., 2012), we also expect other genes than KNOX genes to be regulated by GRFs, and it will be of great interest to gain further insight into these regulatory networks.

**MATERIALS AND METHODS**

**Yeast One-Hybrid Screenings and Domain Mappings**

For yeast (Saccharomyces cerevisiae) one-hybrid screenings, the amplified a ACT-Ze6 cDNA library (constructed with cDNA derived from rice [Oryza sativa ssp. indica ‘IR8’] embryos, 6 d after pollination) described by Postma-Haarsma et al. (1999) was converted into a pACTII-ZEO library by Cre-loxP-mediated subcloning in Escherichia coli BNN132 (Ouwerkerk and Meijer, 2001). All handlings with yeast were as described earlier (Meijer et al., 2000; Ouwerkerk and Meijer, 2001, 2011). Yeast strain Y187 (Clontech, genotypes: MATA, ura3-52, his3-200, ade2-101, trp1-901, leu2-3,112, mat′, gal4Δ, gln5Δ, URA3::GAL1-lacZ, GAL1-lacZ2) derivatives with HIS3 reporter constructs were integrated via the pINT1 integrative vector system at the nonessential locus PDC6 as described earlier (Meijer et al., 1998) and were generated as follows. ProOsK1::HIS3 constructs were based on an earlier described promoter fragment in which the sequence context of the translation start codon was changed to an NcoI restriction site (Postma-Haarsma et al., 2002). Two promoter fragments, a 750-bp Scl–NcoI fragment (in GenBank accession no. AF237878) to construct ProOsK1::HIS3 and a 1,349-bp Sal–NcoI fragment (in GenBank accession no. AF237875) to construct ProOsK1::HIS3 were subcloned into pUC21 (GenBank accession no. AF226341). From the resulting pUC21 derivatives, NorI-Xhol fragments were cloned in NorI/SalI-cut pHS3NX (GenBank accession no. AF226300), leading to promoter fragments of 662 and 1,264 bp fused to HIS3, respectively. Next, NorI-Xhol fragments (containing the ProOsK1::HIS3 fusions) were cloned into NorI/XhoI-cut pNIT1 (GenBank accession no. AF299993). The resulting plasmids were linearized with NcoI and introduced in yeast to generate Y187::ProOsK1-662 and Y187::ProOsK1-1264. One-hybrid screenings were performed with selection on medium lacking Leu and His and containing 5 μg/3-A. Library plasmids were isolated from positive yeast colonies as described earlier (Meijer et al., 2000; Ouwerkerk and Meijer, 2011) and transformed into E. coli DH5α for subsequent sequence analysis (Baseclear) and donings.

A set of control yeast strains was used to study the binding specificity of the clones obtained from the yeast one-hybrid screenings. These control strains were Y187::ProOsK1-1130, Y187::ProOsK1-483, Y187::ProOsK1-1170, and the promoterless HIS3 reporter control strain YP0101 (Ouwerkerk and Meijer, 2001, 2011). The ProOsK1::HIS3 construct was derived from pVDFR767 (kindly provided by Van der Have B.V.), which contains the OsK1 promoter sequence as reported by Matsuoka et al. (1993; GenBank accession no. D16007; ATG start codon at 1,065 bp). From this plasmid, an AluI/BatI-FseI (~521 to ~38) fragment was cloned in EcoRV/FseI-cut pUC21 (GenBank accession no. AF223641) and subsequently subcloned as NotI-EcoRI fragment in pHS3NX (GenBank accession no. AF223600). From the resulting plasmid, a ProOsK1::HIS3 fusion was excised as NorI-Xhol fragment and cloned in NorI/XhoI-cut pNIT1 (GenBank accession no. AF299993). ProOsK3::HIS3 was based on an OsK3 promoter fragment (in GenBank accession no. AF237876) isolated earlier (Postma-Haarsma et al., 2002). A 1,170-bp fragment (directly upstream of the ATG) was subcloned using NotI/XhoI to the same sites in pUC-21 (GenBank accession
no. AF294075). From the resulting plasmid, a NcoI-EcoRI fragment was cloned into the same sites of pBSK(+) and then the whole cassette comprising the Osko3 promoter fused to the H53 gene was cloned into pENTI as NcoI-XhoI fragment.

The short Osko2 promoter was dissected into three fragments, spanning from −753 to −308, −338 to −28, and −328 to −83 with respect to the translation start codon, respectively, using the PCR primer sets 2SNIN/2SN45S, 2SN2N/2SN55S, and 2SN3N/2SN65S (Supplemental Table S1) resulting in fragments of 246 (fragment A), 244 (fragment B), and 246 bp (fragment C), respectively. These were first cloned in pCR2.1-Topo (Invitrogen) for sequence analysis. Next, NcoI-SalI fragments were transferred to NotI/Sall cut pENTI-HISSBN (GenBank accession no. AF016966). A fourth fragment (D) was obtained by annealing the primers Osko2D-Fw and Osko2D-Rev and cloning of the annealed 34-bp fragment D (spanning from −327 to −294) supplied with Sall and NotI overhanging ends into pENTI-HISSBN. From the resulting plasmids, NcoI-SalI or NcoI-AggI fragments were introduced in yeast to generate Y187-ProOsko2-A, Y187-ProOsko2-B, Y187-ProOsko2-C, and Y187-ProOsko2-D.

pACTI constructs expressing Arabidopsis (Arabidopsis thaliana) GRF genes as translational fusion proteins with the Gal4AD were made as follows. For cloning AGRF4, AGRF5, and AGRF6, full-length cDNA clones were assembled from two parts. The N-terminal parts were PCR amplified using Phusion DNA polymerase (Finzymes) on cDNA derived from Arabidopsis (ecotype Columbia) using primer sets AGRF4-Fw/AGR4-Rev, AGRF5-Fw/AGR5-Rev, and AGRF6-Fw/AGR6-Rev (Supplemental Table S1) to introduce a NcoI restriction site at the ATG start codon embedded or, in case of the AGRF5, an EcoRI restriction site just upstream of the starting ATG. The resulting PCR products were ligated into pTOPO2.1 vector (Invitrogen) for sequence analysis and digested with NcoI-PvuII (AGR4F), EcoRI-XcmI (AGR5F), and NcoI-NcoI (AGR6F) for further cloning. The C-terminal domains of the AGRF genes were excised as the PvuII-XhoI fragment from M61B1 (GenBank accession nos. BE522365, and BE522366, AGR4F), the XcmI-XhoI fragment from M61B1 (GenBank accession nos. BE525293, AGR5F), and the NcoI-HindIII fragment from RFLA987-AU3 (GenBank accession no. AF060586, AGR6F). Finally, AGRF N- and C-terminal subfragments were joined together in pACTI between NcoI and XhoI for AGRF4, EcoRI-XhoI for AGRF5, and NcoI-HindIII for AGRF6, respectively.

For DNA-binding mapping assays in yeast, the WRC and QLQ domains from pACTI-AGR5F010 were subcloned into pACTI11a (Meijer et al., 1997) as follows. The fragment containing the WRC domain was amplified by PCR using primers WRC-Fw and WRC-Rev (Supplemental Table S1) and cloned into pCB blueScript II (Stratagene) by EcoRI and XhoI for sequencing and further cloning. From this plasmid, an XhoI-EcoRI fragment was transferred to Sall (partial)/EcoRI-cut pACTI11a resulting in pACTI-WRC. From pACTI-AGR5F010, an NcoI-NotI fragment (containing both QLQ and WRC domains) was cloned into NcoI/NcoI-cut pUC28. From this plasmid, an NcoI-Sall fragment was cloned into pACTI11a and cut with NcoI-XhoI, resulting in pACTI-QLQ-WRC. A fragment containing only the QLQ domain was excised by NcoI-HindIII from the pUC28 clone and was cloned into pACTI11a and digested with NcoI and SpeI, resulting in pACTI-QLQ.

Bioinformatics and Phylogenetic Analysis

BLAST searches of the rice genomic sequence were carried out with the BLAST program running at National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov], TIGR [http://www.tigr.org/], and GRAMENE [http://www.gramene.org]. To predict intron-exon boundaries of the rice and Arabidopsis genes, we used GENSCAN ([http://genes.mit.edu/burgelab/] and the GRAMENE predictions of the Sanger Ensemble. The predicted sequences were adjusted manually based on comparisons with EST or cDNA sequences using Vector NTI10 (Invitrogen). Duplicated amino acid sequences of GRF genes from Arabidopsis, barley (Hordeum vulgare), maize (Zea mays), and rice were used to construct a phylogenetic tree (procedure according to Harrison and Langdale, 2006). The whole protein sequences were aligned using ClustalW (Thompson et al., 1994) at the European Bioinformatics Institute server with default settings and then manually revised using BioEdit (Hall, 1999). The conserved QLQ and WRC domains of 160 amino acids (including caps) were used for phylogenetic study. Phylogenetic trees were obtained by maximum parsimony methods using PAUP, version 4.0b10 (Swofford, 2002). From 1,000 replications, three trees were obtained, and a strict consensus tree was calculated with PAUP, version 4.0b10 (Swofford, 2002). The bootstrap probability of each branch was estimated with 500 replications.

Binary Vector Constructs

All GRF overexpression constructs were made in binary vector pC1301intB-35SnosEX (GenBank accession no. AY560325; Kuijt et al., 2004). OsGRF3 and OsGRF10 overexpression constructs were made by subcloning EcoRI-XhoI fragments from pACTII-OsGRF3 and pACTII-OsGRF10, the original clones obtained in yeast one-hybrid screenings. AGRF4 was overexpressed by cloning an EcoRI-XhoI fragment from clone M25D3. An AGRF5 overexpression construct was made by transferring an EcoRI-XhoI fragment from pACTII-AGR5F. AGRF6 was overexpressed by transferring EST clone pda06900 (GenBank accession no. AY005586), firstly in pUC21 between EcoRI and KpnI and then as EcoRI/XhoI fragment.

To silence GRF genes in rice, an RNAi approach was taken. For this, a binary construct using Gateway technology was made. An attB-flanked PCR product, produced by the amplification of BGRF1 cDNA with attI-specific primers (AtBI BGRF1Fw and AtBI BGRF1Rev; Supplemental Table S1), was introduced in pDONR207 by BP recombination. Subsequently, the RNAi construct was obtained by attI/attI recombination using LR clonase enzyme (Invitrogen) between vector pBio6378 (kindly provided by Biogemma) and available pDONR207 containing the BGRF1 open reading frame. This sequence was cloned in both orientations, with the tubulin intron (1 kb) in the middle allowing the formation of the hairpin that mediates RNAI. To get constitutive silencing, the expression of the transgene was driven by the strong promoter of the Caussia vein mosaic virus.

For expression pattern analysis, a 1,560-bp fragment of the 3′ regulatory sequence of OsGRF10 was amplified with the Expand High Fidelity PCR System (Roche) with primers ProOsGRF10-Fw and ProOsGRF10-Rev (Supplemental Table S1). With the ProOsGRF10-Rev primer, an NcoI restriction site is created around the ATG by changing 2 bp (GAATGG to CCATGG). PCR products were ligated into the pCR2.1-TOPO vector (Invitrogen) for sequence analysis. The correct clone of ProOsGRF10 was digested with Sall and NcoI and cloned into binary vector pCAMBIA1301Z (GenBank accession no. AF234312) for translational fusion to the GLU (εεεε) gene. Prior to plant transformation, binary vectors were transferred from E. coli strain DH5α to Agrobacterium tumefaciens strain LBA4404 (for rice transformation) or LBA1115 (for Arabidopsis transformation) via triparental mating. Ten independent plant lines were made and analyzed for GUS expression using X-Gluc stainings.

Plant Transformation and Growth Conditions

Transformation of japonica rice cultivar Taipe 39 was performed as previ- ously described (Yamada et al., 2003). Rice seeds were disinfested with 0.1 M sodium hypochlorite for 4 min and planted in 10-cm plastic pots containing soil supplemented with 15% peat (v:v) and nystatin, and 100 mg L−1 1 Suc until transfer to the greenhouse, where they were grown at 28°C under a 16-h photoperiod and 85% humidity. With the (shorter) cDNA isolated in the original yeast screens (OsGRF3), seven independent rice overexpressor lines were analyzed, and with the full-length OsGRF3 cDNA, two independent lines were analyzed. Overexpression lines with the shorter cDNA showed similar morphological alterations, although effects of the longer version were more severe. Ten independent overexpression lines harboring the full-length OsGRF10 cDNA construct were analyzed.

In the case of the RNL plants, a transformed A. thaliana (strain EHA105) was cocultivated with rice calli (cv. Nipponbare, japonica rice), and plants were regenerated using methods in Sallaud et al. (2004). A short 35S promoter sequence was inserted to Geneticon was conducted, and positive regenerants were transferred to test tubes. A secondary RT-PCR screening was conducted using primers GRF222Fw and GRF500Rev designed on a conserved DNA binding domain sequence (WRC motif). Selected plants were transferred to soil pots and grown until maturity. Arabidopsis Pro35S:GUS (in Columbia background) and ProKNAT2:GUS (in C24 background) lines were transformed by the floral dip method (Clough and Bent, 1998). Arabidopsis seeds were surface sterilized using chlorosulfonic acid, sown on one-half-strength Murashige and Skoog medium containing 20 mg l−1 hygromycin, 100 mg l−1 nystatin, and 100 mg l−1 timentin, and incubated in the dark at 4°C for 72 h before germination at 21°C under a 16-h photoperiod. Plants 3 to 4 weeks old were transferred to soil and grown at 21°C under a 16-h photoperiod and 60% relative humidity.

EMSA and Constructs for Expression of Recombinant GRF Proteins

OsGRF3 and OsGRF10 cDNA fragments were fused in frame with the GST sequence in expression vector pGEX-KG (Guan and Dixon, 1991) by subcloning EcoRI-XhoI fragments from the pACTII clones isolated in the library screening. pGEX-AGR4F and pGEX-AGR5F were made by subcloning NcoI-XhoI fragments from the respective pACTII plasmids between NcoI and XhoI of pGEX-KG. pGEX- AGR5F was made by cloning an EcoRI-XhoI fragment from pACTIB-AGR5F.
between EcoRI and XhoI of pGEX-KG. To extract recombinant protein, 5 ml of overnight cultures of BL21DE3/plys cells (Novagen) carrying pGEX plasmids were used to inoculate 500 ml Luria-Bertani medium containing 200 mg l⁻¹ carbencillin and 25 mg l⁻¹ chloramphenicol and grown at 37°C to an optical density at 600 nm of 0.5. Next, protein synthesis was induced by the addition of isopropylthio-β-galactoside to final concentration of 1 mM, and cultures were grown for 4 h at 29°C. The harvested cells were suspended in 20 ml phosphate-buffered saline (PBS) and frozen in liquid nitrogen. After thawing on ice, the bacteria were lysed by sonication (eight times, 10 s burst; 5 s pause between bursts) and centrifuged at 18,000 rpm for 30 min at 4°C, and the supernatant was filtered through a 0.45-μm membrane. Protein purification was performed using Poly-Prep Chromatography columns (Bio-Rad 731-1530) containing 0.5 ml settled Glutathione-Sepharose 4B beads (Amersham Biosciences). Columns were first washed two times with 10 ml PBS before bacterial extract was passed through. After binding, columns were washed with 10 ml PBS, and bound proteins were eluted in 2.5 ml (10 x 0.25 ml) glutathione elution buffer (100 mM glutathione, 500 mM Tris-HCL, pH 8.0). Eluted protein was concentrated using Microcon centrifugal filter devices (Millipore) according to manufacturer’s protocol, and the protein content was determined by the method of Bradford. All EMSA reactions contained 100 ng poly-(dC-dC)poly-(dI-dC); Amersham Pharmacia) and 0.5 ng of 5'end-labeled probe (approximately 10⁸ cpm μg⁻¹) in 22.5 μl nuclear extraction buffer (Green et al., 1987) supplemented with 75 mM KCl. Probes used in labelings originated from combinations of annealed oligonucleotides (primers Osk2d2-Fw and Osk2d2-Rev; Supplemental Table S1) or gel-purified PCR reactions using primer sets 2SN1/2SN4, 2SN2/2SN5, and 2SN3/2SN6 (Supplemental Table S1) resulting in fragments A, B, and C, respectively, to the fragments used in yeast strains Y187:pOskn2-A to Y187:pOskn2-C. DNA-binding reactions were loaded on 4% (w/v) acrylamide/bisacrylamide (37.5:1) gels with the current switched on (10 V cm⁻¹). The gels were dried on Whatman DE81 paper on top of Whatman 3MM paper and autoradiographed.

Histological and Morphological Observations
Arabidopsis and rice tissues were stained for GUS expression by incubation of the samples overnight at 37°C in 100 mM sodium phosphate buffer, pH 7.5-7.7, 10 mM sodium EDTA, 0.5 mM potassium ferricyanide, 0.1% Triton X-100, and 2 mM X-Gluc. The reaction was stopped by adding 70% ethanol for direct observations using a Leica MZ12 stereomicroscope equipped with a Sony 3CCD camera (DKC 5000). For more detailed analysis, 70-μm fresh vibratome sections (made with a Leica VT1000S vibratome microtome) were incubated in prechilled (~2°C) 90% acetone for 1 h at ~20°C. The samples were then stained overnight under the same reaction conditions as above. Phenotypes of Arabidopsis plants in tissue culture were observed and photographed using a Leica MZ12 stereomicroscope equipped with a Sony 3CCD camera (DKC 5000), and greenhouse-grown Arabidopsis and rice plants were photographed with a Nikon Coolpix 5000 camera.

Transient Expression Analysis in Onion Cells
Particle bombardment assays were performed on the abaxial side of adaxial epidermal peels from onion (Allium cepa) bulb scales as described earlier (Kuitt et al., 2004).

Transient Expression Using A. tumefaciens-Mediated Transformation of Rice Calli
Transformation of calli derived from ProOsk2::GUS seeds (Postma-Haarsma et al., 2002) was as previously described by Scarpetta et al. (2000) using A. tumefaciens strain LBA 4404 harboring OsGFR3 and OsGFR10 constructs driven by the CaMV 35S promoter. Two independent transgenic ProOskn2::GUS lines were used for the transformation experiments, and a ProCaMV 35S:GFP overexpression construct was used in parallel transformations as control. In brief, embryonic calli were induced on scutella by growing germinating seeds on callus induction medium with the CaMV 35S promoter. Two independent transgenic ProOskn2:GUS lines were used for transformation experiments. Transformation of calli derived from ProOskn2:GUS seeds (Postma-Haarsma et al., 2002) was as previously described by Scarpetta et al. (2000) using A. tumefaciens strain LBA 4404 harboring OsGFR3 and OsGFR10 constructs driven by the CaMV 35S promoter. Two independent transgenic ProOskn2::GUS lines were used for the transformation experiments, and a ProCaMV 35S:GFP overexpression construct was used in parallel transformations as control. In brief, embryonic calli were induced on scutella by growing germinating seeds on callus induction medium (Basal R2 medium supplemented with 10 g l⁻¹ Glc, 2.5 mg l⁻¹ 2,4-Dichlorophenoxyacetic acid, 100 μM acetosyringone, pH 5.2), calli were transferred to plates with cocultivation medium for 3 d. Following cocultivation, selection of transgenic calli was performed on Basal R2 medium supplemented with 30 g l⁻¹ Suc and 2.5 mg l⁻¹ 2,4-Dichlorophenoxyacetic acid together with 100 mg l⁻¹ hygromycin, 100 mg l⁻¹ cefotaxime, and 100 mg l⁻¹ vancomycin to prevent overgrowth by A. tumefaciens. All binary overexpression constructs were based on the hygromycin phosphotransferase-intron vectors, and the presence of the intron in the hygromycin phosphotransferase gene renders the A. tumefaciens strain sensitive toward hygromycin. Transformed embryonic ProOskn2::GUS calli grown on cocultivation medium or selection media were collected at several time points after inoculation with A. tumefaciens, stained for GUS expression with X-Gluc as indicated in Supplemental Figure S10, and photographed. The photos were opened in ImageJ and split into blue, red, and green channels. Next, the GUS density was measured as the negative of the average pixel value in the red channel over single calli.

Rice Protoplast Isolation and Transient Expression Assays with GUS and Firefly Luciferase (FLUC) Reporters
Preparation of rice protoplasts, transfection with effector/reporter gene constructs, and protein extraction for GUS and FLUC assay measurements were according to Chen et al. (2006) or to the manufacturer’s instructions (Promega). One hundred rice seedlings (cv IR64, indica rice) were grown in soil (28°C, 85% humidity, and 12-h dark and 12-h light) in a 10-cm-diameter pot for about 12 to 14 d (3 d in the dark, and the last 10–11 d under light/dark). Plantlets were collected and kept wrapped in aluminum foil on ice. Using razor blades, stems and leaves were cut into approximately 0.5 mm pieces, which were collected in 50 ml centrifuge tubes with 25 ml Enzyme Solution (containing 1.5% cellulase and 0.3% Macerozyme; see Chen et al., 2006). Further handlings to prepare protoplasts and transformation with effector and reporter gene constructs were also according to Chen et al. (2006). Usually, about 10⁷ cells were obtained from 100 rice seedlings, and 200 μl (1.5 to 2.5 x 10⁷ cells ml⁻¹) of suspended protoplasts were used for transfection with 4 μg effector plasmid, 5 μg effector plasmid, and 3 μg FLUC plasmid.

Plasmid FLUC is the firefly luciferase gene driven by the CaMV 35S promoter and B leader (Ossato et al., 2010) and was added as a control for transformation efficiency. Activity of the effectors was derived as GUS activity normalized on FLUC activity. The effector construct with OsGFR10 is the same transcription fusion with the CaMV 35S promoter as in the binary vector construct used to make stable overexpression plants but was cloned in the high copy vector SK1 BlueScript II (Strategene). The effector gene OsUbiII, in which the effector gene is driven by the constitutive maize Ubiquitin promoter, and the reporter gene constructs K373+305 bp are described in Ossato et al. (2010). ProUbi:BGFR1 was cloned by coupling the BGFR1 cDNA to the maize Ubiquitin promoter and the Nos terminator in vector pCBRII-TOPO (Invitrogen).

Protoplasts were obtained as follows. After overnight incubation, the protoplasts were resuspended by gently pipetting up and down and transferred to 2 ml Eppendorf tubes, kept on ice, and centrifuged at 16,000 rpm for 10 min at 4°C. Next, the supernatant was removed and 100 μl Cell Culture Lysis Reagent (Promega) buffer was added to the protoplast pellet. Cells were shared by pipetting up and down, vortexed, and kept on ice. Next, the tubes were centrifuged at 16,000 rpm for 10 min at 4°C, and the supernatant with the protein extracts was transferred to 1.5-ml tubes and kept on ice until the FLUC assays were ready and then frozen immediately with liquid nitrogen and stored at −80°C.

Detection of GUS activity was essentially according to Jefferson (1987). Twenty microliters cell lysate was incubated at 37°C with 180 μl 4-methylumbelliferyl-β-D-glucuronide for the fluorimterical assay. Ten-microliter samples of the reaction mixes were taken at 3 and 6 h, added to 190 μl 0.2% Na₂CO₃ to stop the reaction, and measured for 4-methylumbelliferone fluorescence against a 4-methylumbelliferone calibration series in a Cytofluor 2350 fluorimeter (Millipore).

For detection of FLUC activity, 10 μl cell lysate was transferred in duplicate to white plates for luminescence assays containing 90 μl Cell Culture Lysis Reagent, and, next, 100 μl of Bright-Clo Luciferase Assay buffer (Promega) was added. The reactions were immediately read during 5 s in a Perkin Elmer Victor 3 with 10-min intervals.

SEM
Tissue was fixed overnight at room temperature in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Fixed tissue was dehydrated in a graded acetone series and critical point dried with CO₂. Specimens were coated with a thin layer of gold using a Polaron E5100 sputter coater and were examined with a JEOL 6400 SEM with a tungsten filament operating at an accelerating voltage of 5 kV.

RT-PCR
Total RNA extraction from rice materials was performed using TriZol according to the manufacturer’s instructions (Invitrogen). Genomic DNA...
contaminants were removed from RNA samples by incubating with DNA-free (Ambion) at 37°C for 30 min according to the manufacturer’s instructions. First-strand cDNA was synthesized starting from 1 μg of total RNA using primer B26 with SuperScript III reverse transcriptase (Invitrogen) as described by the manufacturer. RT-PCR reactions were performed on single-strand cDNA using primer sets Oskn2-K4-5/Oskn2-K4-6 for Oskn2, OsGRF3-Fw2/OsGRF3-Rev2 for OsGRF3, and OsGRF10-Fw3/OsGRF10-Rev4 for OsGRF10 (Supplemental Table S1). The concentrations of the cDNA samples were equalized using actin primers OsActin U/OsActin L (Momena et al., 2002). Aliquots of the amplified products were separated on 1% Tris-borate/EDTA and 1% agarose gels, blotted on Hybond N+ filters, and hybridized to Oskn2, OsGRF3, and OsGRF10 probes. qPCR for Oskn2 mRNA in OsGRF3 Overexpression seedlings was done by using the SYBR Green I Master Mix and Light Cycler 480 (Roche) with primers Oskn2-qFw and Oskn2-qRev. Normalization of Oskn2 expression was done by using primers OsLhi-Fw and OsLhi-Rev to amplify the ubiquitin gene (Supplemental Table S1).

qPCR Experiments

T2 and T3 rice plants were grown under 12-h light/12-h dark at 28°C in a growth chamber, together with the wild type (cv Taipei 309 for OsGRF3 overexpressors and cv Nipponbare for RNAi GRF lines). RNA was extracted from pools of five plants by using TRIzol (Invitrogen) and treated with DNaseI RNase free (Ambion), and 1 μg was retrotranscribed with oligo(dT) and SuperScript III (Invitrogen). The expression levels of genes of interest were monitored by qPCR using SYBR Green I Master Mix and Light Cycler 480 (Roche). Primers used were GRF3Fw, GRF3Rev, GRF4Fw, GRF4Rev, GRF5Fw, GRF5Rev, Oskn1Fw, Oskn1Rev, Oskn2-1803Fw, Oskn2-1805Rev, Oskn3Fw, and Oskn3Rev. The OsLhi gene was used to normalize data. Experiments were performed with three biological replicates, of which each was performed in three technical replicates.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Yeast one-hybrid assays with OsGRF3 and OsGRF10 expression constructs and rice promoter knox constructs.

Supplemental Figure S2. Interaction of OsGRF3 and OsGRF10 proteins with Oskn2 promoter subfragments B, C, and D and DNA-binding properties of the QLQ and WRC domains.

Supplemental Figure S3. Interaction of OsGRF3 and Arabidopsis GRF proteins with the Oskn2 promoter and subfragments.

Supplemental Figure S4. Expression pattern analysis of OsGRF10 in rice with promoter-GUS fusion constructs and nuclear localization of OsGRF10.

Supplemental Figure S5. Node numbers, internode lengths, and total plant lengths of OsGRF3 and OsGRF10 overexpression plants.

Supplemental Figure S6. Unrooted phylogenetic tree showing the predicted relationship between GRF proteins from rice, maize, barley, and Arabidopsis.

Supplemental Figure S7. Chromosomal positions of the GRF genes on the 12 rice chromosomes.

Supplemental Figure S8. Alignments of the full-length GRF protein sequences from rice (OsGRF), maize (ZmGRF), barley (BcGRF1), and Arabidopsis (AtGRF).

Supplemental Figure S9. Intron-exon organization of rice and Arabidopsis GRF genes.

Supplemental Figure S10. Effects of OsGRF3 and OsGRF10 overexpression on ProOsokn2::GUS in rice calli.

Supplemental Figure S11. Effects of ectopic expression of rice and Arabidopsis GRF genes on ProKNAT2::GUS expression and SAM development in Arabidopsis.

Supplemental Table S1. Overview of all oligonucleotides used in this paper.

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LITERATURE CITED


Interactions between GROWTH-REGULATING FACTOR and Homebox Genes


