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OsARF1, an auxin response factor from rice, is auxin-regulated and classifies as a primary auxin responsive gene

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Abstract

We screened for auxin-induced genes with an expression correlated to the auxin-induced growth response from rice coleoptiles by fluorescent differential display. A rice homologue of the auxin response factor (*ARF*) family of transcriptional regulators, *OsARF1*, was identified. An OsARF1:GFP fusion protein was localized to the nucleus. Steady-state levels of *OsARF1* mRNA correlated positively with auxin-dependent differential growth: gravitropic stimulation enhanced the amount of *OsARF1* transcript in the lower, faster-growing flank accompanied by a decrease in the upper flank of gravitropically stimulated rice coleoptiles. Exogenous auxin up-regulated the steady-state level of *OsARF1* mRNA within 15–30 min. This up-regulation is independent of *de novo* protein synthesis. Thus, *OsARF1* is the first *ARF* that classifies as an early auxin-responsive gene. The observed auxin-dependent regulation comprises a new level of regulation in auxin-induced gene expression and is discussed as a possible feedback mechanism in plant growth control.

Introduction

Plants survive because they are able to sense environmental conditions and react appropriately by growth and differentiation. Environmental cues such as light and gravity trigger a differential growth response, often resulting in the modification of morphology. Early studies on gravitropism revealed that the perception of the gravitropic stimulus and the resulting growth reaction are spatially separated (Darwin 1880). The search for the transmitting signal led to the discovery of the first plant hormone, auxin, characterized in graminean coleoptiles (Went, 1929). Later the gravitropic and phototropic reactions of the coleoptile were explained by the Cholodny-Went theory in terms of a lateral redistribution of the basipetal auxin flow, resulting in an auxin concentration gradient that causes the observed difference in growth (Cholodny, 1927; Went, 1929).

What are the molecular events linking increased auxin concentrations with the elevated growth of plant tissues? Numerous genes, such as the *SAUR* (McClure *et al.*, 1989), *GH3* (Hagen *et al.*, 1984) and *Aux/IAA* (Theologis *et al.*, 1985) genes, are rapidly and specifically induced by auxin. Several members of these gene families are primary auxin-responsive genes, since their induction is rapid and does not require *de novo* protein synthesis (Theologis *et al.*, 1985; McClure *et al.*, 1987; Abel and Theologis, 1996; Reed, 2001). Auxin responsiveness is conferred to several of these genes by conserved promotor elements, termed 'auxin-responsive elements' (AuxRE) (Ulmasov *et al.*, 1995).

AuxRE promotor elements are bound by a new class of plant-specific transcription factors, auxin response factors (ARFs), identified in *Arabidopsis* (Ulmasov *et al.*, 1997a). ARFs consist of an amino-terminal DNA-binding domain and most ARFs con-

A partial rice cDNA (accession no AB071300) corresponding to the genomic sequence BAA92916.1 (referred to as 'OsARF2' in this study) has been isolated recently and designated OsARF16 (Sato Y *et al.*, 2001). Auxin response factor family in rice. Genes Genet. 76: 373–380.

tain a C-terminal region with two conserved domains that are involved in homo- and heterodimerization (Kim *et al.*, 1997; Ulmasov *et al.*, 1997a; Ulmasov *et al.*, 1999b). In *Arabidopsis*, the ARF gene family consists of 23 members and some of them have been shown to be able to repress or to activate expression of reporter genes with an AuxRE promotor element (Ulmasov *et al.*, 1999a). The importance of ARFs for normal development and growth is emphasized by the identification of ARF mutants in *Arabidopsis* with severe defects in specific auxin-mediated processes: the ARF5/MONOPTEROS mutant is affected in early embryo development and vascular strand formation (Hardtke and Berleth, 1998). ARF3/ETTIN mutants show defects in floral patterning (Sessions *et al.*, 1997; Nemhauser *et al.*, 2000), and ARF7/NPH4 mutants have defects in photo- and gravitropic responses accompanied by changes in expression of auxin-induced genes (Watahiki and Yamamoto, 1997; Stowe-Evans *et al.*, 1998; Harper *et al.*, 2000).

We applied a fluorescent differential display (FDD) method (Ito *et al.*, 1994; Kuno *et al.*, 2000) to identify genes involved in the regulation of auxin-controlled growth in rice coleoptiles. The coleoptile exhibits a physiologically well-studied, auxin-controlled growth response that can be triggered by environmental factors such as light and gravity and is only due to cell elongation (Wada, 1961; Pjon and Furuya, 1967; Iino, 1995). When the auxin-producing tip is removed, elongation of the coleoptile depends on the external supply of auxin. Incubation of auxin-depleted coleoptile segments in auxin solution and in water thus provides a simple assay to identify auxin-induced genes. As we were interested in identifying genes involved in the auxin-dependent growth control of the coleoptile, the gene expression pattern of a rice mutant line, *Yin-Yang*, that exhibits a higher auxin-responsiveness of coleoptile growth (Wang and Nick, 1998) was examined in parallel with the wild type. Using FDD, we screened for genes that were (1) specifically induced by auxin in auxin-vs. water-incubated coleoptile segments depleted in internal auxin, and (2) at the same time were differentially expressed in the mutant compared with the wild-type. By including the *Yin-Yang* mutant in our screen we wanted to be able to isolate genes that are not only auxin-induced, but also correlate in their expression with the amplitude of the growth response.

We report here the isolation and characterization of a newly identified auxin response factor, *OsARF1*, a rice homologue to the *Arabidopsis* ARF family of transcription factors. *OsARF1* expression levels positively correlate with the growth response of coleoptiles after application of IAA and are elevated in the faster growing flank of gravitropically stimulated rice coleoptiles. This study shows for the first time that steady-state mRNA levels of an auxin response factor, *OsARF1*, are regulated by auxin. Furthermore, *OsARF1* can be classified as a primary auxin responsive gene, a class of genes that is auxin-induced even in the absence of *de novo* protein synthesis and likely to play an important role in mediating the growth-stimulating effect of auxin. The potential role of OsARF1 as a transcription factor regulating the expression of genes important for growth is discussed.

Materials and methods

Plant material and treatments of seedlings and coleoptiles

Wild-type rice and the *Yin-Yang* mutant line of the same cultivar (*Oryza sativa* L. ssp. *japonica* cv. Nihonmasari) were used throughout the experiments (Wang and Nick, 1998). Seeds were sown on floating plastic meshes in polyacryl boxes stored in light-tight boxes in a dark room at 25 °C. Six days after germination, the apical 2 mm of the coleoptile was removed and subapical segments of 10 mm length used for further experiments. The coleoptile segments were incubated in H_2O, under continuous rotation, for 1 h to wash out endogenous auxin. Subsequently, the sections were incubated in H_2O or indole-3-acetic acid, Fluka, respectively.

For the measurement of elongation growth, about 30 coleoptile segments were cut to an exact length of 10 mm after the incubation in H_2O and the length of the segments was measured at different time points after the onset of incubation in 5 μM auxin.

For gravitropic experiments, seedlings were attached to plastic strips by gluing their caryopses to the strip 4 days after germination in green safelight. After 24 h, coleoptiles were perfectly straight and were gravitropically stimulated by tilting the plastic strips by 90 °C. After 15 or 180 min, the apical 2 mm of the coleoptile were removed and subapical coleoptile segments of about 10 mm length were split into their upper and lower half and harvested separately into liquid nitrogen.

Fluorescent differential display (FDD)

The FDD procedure was essentially the same as described by Kuno *et al.* (2000), except that total RNA was isolated from rice coleoptile segments with a Phenol/SDS/LiCl method (Nagy *et al.*, 1988). Total RNA preparations were treated with RNase-free DNase (Ambion, Austin, TX) for 30 min to remove contaminating genomic DNA. First-strand synthesis was performed with 2.5 μg of total RNA using a Texas Red-labeled 3′-anchored oligo(dT) primer (5′-Texas Red-GT$_{15}$G-3′; Youkigouseikagaku, Tokyo) and the Superscript Preamplification System (Gibco-BRL, Rockville, MD). cDNAs produced from 25 ng of total RNA were amplified by PCR with combinations of the Texas Red-labeled anchored primer and arbitrary 10-mer primers (Kit B, D, F and X, Operon Technologies, Alameda, CA). The PCR conditions were as follows: 94 °C for 3 min, 40 °C for 5 min and 72 °C for 5 min, followed by 24 cycles of 94 °C for 15 s, 40 °C for 2 min, and 72 °C for 1 min, with an additional incubation at 72 °C for 5 min. Electrophoresis and detection of the PCR products was performed with an automated DNA sequencer (SQ5500, Hitachi, Tokyo, Japan).

Cloning of cDNAs of interest

The cDNAs of interest were isolated by preparative electrophoresis and cut out from gels as described by Kuno *et al.* (2000). cDNAs eluted from the excised gel slices were reamplified by PCR with the appropriate primer pairs and PCR products subcloned into the pGEM-T vector (Promega). For each amplified fragment, plasmids of several independent *Escherichia coli* clones were isolated and inserted fragments from these clones were amplified by PCR. Using a fluorescent DNA sequencer, the sizes of reamplified PCR products were confirmed to be identical with the sizes of the original cut-out bands of the FDD-PCR products. Several independent clones with fragments of the correct size were sequenced. The complete cDNA sequence of *OsARF1* was isolated by reverse transcription PCR using gene–specific primers and the '5′-RACE (rapid amplification of cDNA ends) system' (Gibco-BRL).

Isolation of RNA and northern blot analysis

Total RNA was isolated from rice coleoptiles by phenol/chloroform extraction according to Ehmann *et al.* (1991). Total RNA (15 μg) was separated by electrophoresis in agarose-formaldehyde gels and transferred to positively charged nylon membranes (Boehringer, Mannheim, Germany). Hybridization was performed with Rapidhyb hybridization buffer (Amersham Pharmacia) at 65 °C with a γ-[^{32}P]-dCTP random-labeled probe corresponding to *OsARF1* cDNA position 548–2421 or with a *OsARF2* probe (cDNA position 1131–1470). After washing with 2× SSC, 1× SSC and 0.5× SSC washing solutions (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) containing 0.2% (w/v) SDS at 63 °C, membranes were autoradiographed for 16–96 h using an intensifying screen (Biomax, Kodak, Rochester, NY). To confirm equal transfer of RNA onto the membrane, the *OsARF1*/*OsARF2* probe was removed after autoradiography and the membrane re-hybridized with a probe corresponding to rice 28S rRNA.

Transient transformation of tobacco protoplasts with GFP constructs

To assess the intracellular localization of the OsARF1 protein, the full-length *OsARF1* cDNA was inserted in-frame between the translation start signal ATG and the cDNA of a soluble modified green fluorescent protein of the smGFP4 plasmid vector (Davis and Vierstra, 1998). Tobacco protoplasts derived from a tobacco VBI-0 cell culture (Petrašek *et al.*, 1998) were transformed with the smGFP or with the *OsARF1:smGFP* plasmid, respectively, by electroporation (0.4 cm cuvette; 320 V, 125 μF, $\infty\Omega$; using a 'Gene Pulser II'; Biorad, Hercules, CA) as described in Kircher *et al.* (1999). Transformed protoplasts were incubated in darkness for 12–16 h at 25 °C and GFP fluorescence was analyzed by fluorescence microscopy using a standard fluoroescein isothiocyanate filter set.

Results

Differential display screen for putative regulators of auxin-controlled growth

Subapical coleoptile segments from wild-type and the *Yin-Yang* mutant were depleted in internal auxin, and incubated for 1 h in H_2O or a 5 μM solution of the natural auxin, indole-3-acetic acid (IAA), respectively. The *Yin-Yang* mutant exhibits an auxin-induced growth response that is increased by about 50% as compared with the wild-type (Wang and Nick, 1998). After isolation of total RNA from these four samples,

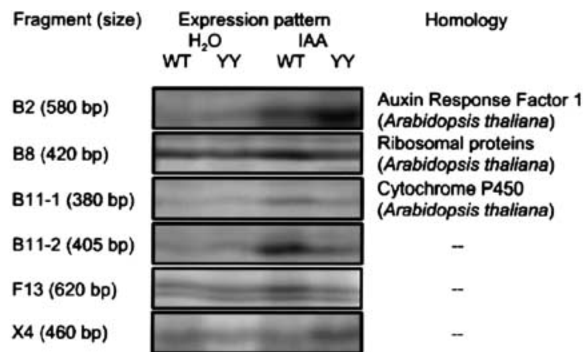

Figure 1. cDNA fragments isolated in the FDD-screen for auxin-regulated genes in rice coleoptile segments. The regions of different FDD gel images shown depict the typical expression pattern of the respective cDNAs.

mRNA fingerprints were generated by FDD. Comparison of auxin-incubated and control incubations of the wild-type and the *Yin Yang* mutant identified bands that (1) were expressed differentially in an auxin-dependent manner, and (2) exhibited a difference in auxin-induced expression between the wild-type and the mutant.

Using 80 different arbitrary decamer primers in the PCR amplification, the expression of about 10 000 mRNAs was visualized by FDD and compared among the four samples. FDD analysis of candidate bands was performed at least three times with total RNA from independent experiments. Of the 10 000 cDNA bands analyzed, about 200 were up- or down-regulated in response to auxin. Among these 200 bands, we identified 6 bands that were differentially expressed between the mutant and wild-type upon auxin incubation, correlating in their transcript level with the magnitude of auxin-induced elongation (Figure 1). In the *Yin-Yang* mutant, two of the six candidate bands showed a higher auxin-induced intensity and four a reduced intensity as compared to the wild-type. Fragments with homologies to the *Arabidopsis* auxin response factor 1, to a ribosomal protein and a cytochrome P450 were isolated (Figure 1). Three of the six candidate cDNAs cloned showed no apparent homology to known DNA sequences in the databases (as assessed with the BLAST homology search; Altschul *et al.*, 1990), probably due to the fact that parts of the isolated FDD fragments consist of the 3′-untranslated region of the respective genes. As the sequence homology of fragment ‘B2’ to an *ARF* suggested a role for the corresponding gene product in the regulation of auxin-dependent gene expression, we isolated the full-length cDNA of this gene and began the characterization of its regulation during the auxin-controlled growth of the rice coleoptile.

Identification of OsARF1*, an auxin response factor from rice*

Application of the 5′-RACE method and screening of a genomic rice (BAC) library led to the identification of the complete cDNA sequence for the isolated *OsAFR-1* cDNA fragment (Figure 2; GenBank accession number AJ306306). The deduced amino acid sequence revealed the closest homology (with 44.2% identity at the amino acid level) to ARF1 (Ulmasov *et al.*, 1997a), an auxin response factor from *Arabidopsis* (Figure 2). The isolated rice gene was therefore termed ‘*Oryza sativa* auxin response factor 1’ (*OsARF1*). Recently, a genomic sequence from rice was deposited in the databases (GenBank accession number BAA92916.1), which shows about 36.5% overall identity to OsARF1 at the amino acid level and hereafter is referred to as OsARF2.

OsARF1 shows the typical features of auxin response factors, such as an N-terminal DNA-binding domain and two C-terminal domains, ‘Box III’ and ‘Box IV’, which are implicated in protein dimerization (Figure 2). The middle region of the OsARF1 protein is rich in proline, serine and threonine residues (Figure 2), a feature shared by ARF1, which can act to represses auxin-induced, AuxRE-mediated gene expression (Ulmasov *et al.*, 1999b). In contrast, activating ARFs from Arabidopsis (such as ARF5-8) contain glutamine-rich stretches (Ulmasov *et al.*, 1999b) that are not present in the OsARF1 middle region.

OsARF1 protein is localized to the nucleus

Sequence homology of OsARF1 to ARF1 suggests that OsARF1 has a function in controlling the transcription of genes with AuxRE in their promoters. Therefore, a nuclear localization of the OsARF1 protein would be expected. Though no typical nuclear localization signal could be identified in OsARF1, there is some sequence similarity to a putative NLS in the ARF1 sequence (Ulmasov *et al.*, 1997a), as indicated in Figure 2. To assess a nuclear localization of OsARF1, protoplasts were prepared from tobacco VBI-0 cells, and transiently transformed with *OsARF1*:*GFP* fusion constructs. The OsARF1:GFP fusion protein was observed to be localized to the nucleus *in vivo* (Figure 3D), whereas in the control experiments the GFP construct without the *OsARF1* cDNA was distributed homogeneously throughout the cell (Figure 3B).

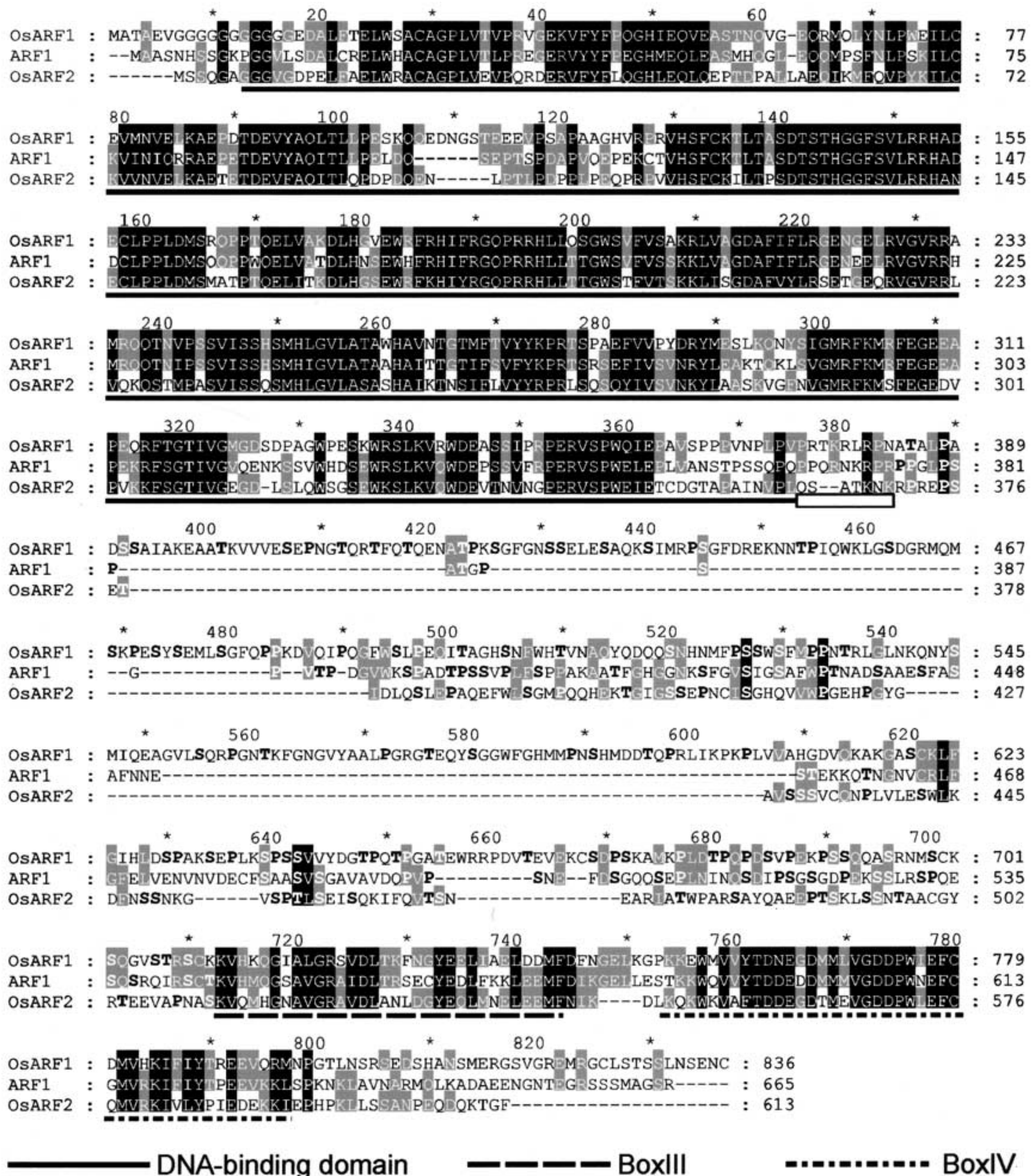

Figure 2. Alignment of the deduced amino acid sequences of OsARF1, OsARF2 and *Arabidopsis* ARF1. OsARF2 is a rice ARF homologue deduced from a rice genomic sequence (Genbank accession number BAA92916.1). The alignment was performed with the Clustal W1.8 program, using standard settings (Thompson *et al.*, 1994) Residues conserved among all three or among two of the three sequences are shaded in black or gray, respectively.

Underlined are the DNA-binding domain and protein-protein interaction domains Box III and Box IV. Sequence identities between OsARF1 and ARF1 in these domains are 80%, 76% and 85%, respectively. Overall identity between the complete amino acid sequences of OsARF1 and ARF1 is 44.2%, of OsARF1 and OsARF2 36.5%. A putative nuclear localization sequence of ARF1 (Ulmasov *et al.*, 1997a) is indicated by a box. Proline (P), serine (S) and threonine (T) residues in the middle region are depicted in bold letters.

The OsARF1 protein therefore contains sequence information conferring nuclear localization to the fusion protein.

OsARF1 *mRNA levels are rapidly induced by auxin and correlate with the growth response*

How does the expression of *OsARF1* correlate with the characteristic auxin-induced growth response of coleoptiles? The induction of coleoptile elongation follows a characteristic bell-shaped dose-response relation with a maximum between 1 and 10 μM auxin for rice (Wang and Nick, 1998). Total RNA was harvested from coleoptile segments that had been incubated in different auxin concentrations after depletion of internal auxin. Then, the abundance of *OsARF1* mRNA was determined in northern blot experiments (Figure 4): the highest level of *OsARF1* transcript was detected after incubation with either 1 or 3 μM auxin, whereas incubation in 100 μM auxin produced low *OsARF1* transcript levels, comparable to that obtained upon incubation in water. *OsARF1* mRNA steady-state levels therefore follow an optimum curve with a maximum of between 1 and 3 μM auxin, comparable to the growth curve of coleoptile segments (Wang and Nick, 1998).

The auxin response of coleoptile segments that have been depleted in internal auxin occurs from about 15 min after the addition of auxin, thus representing one of the fastest known responses to auxin (Evans and Ray, 1969). When rice coleoptile segments are incubated in 5 μM auxin (the optimal concentration for inducing elongation (Wang and Nick, 1998)), coleoptile length increases significantly, starting between 15 and 30 min after the addition of auxin (Figure 5 A, B). *OsARF1* transcript levels increase 15–30 min after the onset of incubation (Figure 5C), and remain high for at least two hours of auxin incubation (Figure 5C), concomitant with a high growth rate during this period.

OsARF1 *is an early auxin-responsive gene*

The rapid induction of *OsARF1* mRNA steady-state levels observed in response to auxin incubation of rice coleoptile segments (Figure 5) raised the question whether *OsARF1* might be an early auxin-responsive gene. Beside a fast induction, early auxin-responsive genes are characterized by an induction of mRNA accumulation even when *de novo* protein synthesis is blocked.

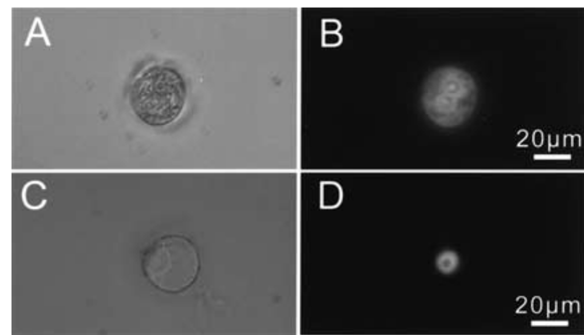

Figure 3. *In vivo* localization of an OsARF1:GFP fusion protein in tobacco protoplasts. Tobacco protoplasts were transformed with a *GFP* construct **(A and B)** as a control, and with a *OsARF1:GFP* fusion construct **(C and D)**. A and C are the bright-field images corresponding to the epifluorescence images B and D, respectively.

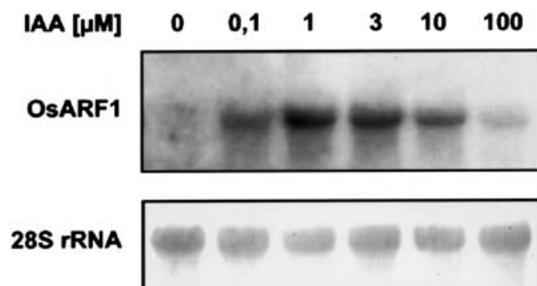

Figure 4. Auxin concentration dependence of *OsARF1* expression. *OsARF1* expression in rice coleoptile segments depleted of internal auxin and incubated in different concentrations of indole-3-acetic acid for 60 min was analyzed in northern blot experiments. The blot prepared with 15 μg of total RNA was hybridized with an *OsARF1* probe (upper panel), and the same membrane rehybridized with a rice 28*S* rRNA probe (lower panel).

We tested if *OsARF1* induction depends on *de novo* protein synthesis by incubating coleoptile segments in IAA, the protein synthesis inhibitor cycloheximide (CHX), or both. Pre-incubation of coleoptile segments with CHX did not prevent the auxin-induced accumulation of *OsARF1* transcript (Figure 5D, lane 4). CHX treatment alone, as well as auxin treatment, lead to an increase in *OsARF1* transcript (Figure 5D, compare lanes 2 and 3 to lane 1). CHX pretreatment and auxin incubation together (lane 4) led to a super-induction of *OsARF1* transcript.

Gravitropic stimulation induces a gradient of the OsARF1 *transcript level across the coleoptile*

Experiments with coleoptile segments revealed a correlation between exogenous auxin and the steady-state level of *OsARF1* mRNA (Figures 4 and 5). This raised the question whether *OsARF1* expression can be ma-

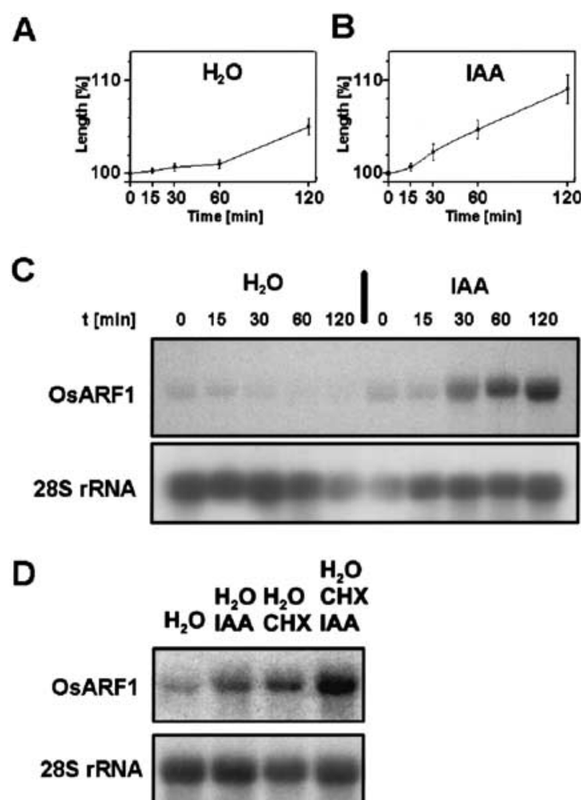

Figure 5. Auxin-induced growth response of rice coleoptile segments and *OsARF1* mRNA steady-state levels upon auxin incubation and inhibition of *de novo* protein synthesis. Elongation kinetics of rice coleoptile segments incubated in H_2O (A) or 5 μM indole-3-acetic acid (B) after washing out of internal auxin for 60 min. (C) Northern blot from total RNA (15 μg) from rice coleoptile segments incubated as described for A and B. The blot was hybridized with probes for *OsARF1* (upper panel) and the same membrane rehybridized with a rice 28*S* rRNA probe (lower panel). (D) The effect of *de novo* protein synthesis inhibition on auxin-induced expression of *OsARF1* was analyzed by northern blot. Coleoptile segments incubated 120 min in H_2O (lane 1), 60 min in H_2O plus 60 min in 5 μM auxin (lane 2), 30 min H_2O plus 90 min 70 μM cycloheximide (lane 3), and 30 min H_2O plus 30 min 70 μM cycloheximide plus 60 min 70 μM cycloheximide and 5 μM Auxin (lane 4). The membrane was hybridized with a *OsARF1* probe (upper panel), and the same membrane rehybridized with a rice 28S rRNA probe (lower panel).

nipulated by stimuli that alter the endogenous level of auxin. According to the Cholodny-Went theory, both gravitropism and phototropism are caused by a lateral transport of active auxin produced in the tip and transported basipetally to the elongating regions of the coleoptile (Cholodny, 1927; Went, 1929). The difference in auxin concentration between the coleoptile flanks is thought to cause the observed growth gradient that drives the bending reaction of the coleoptile.

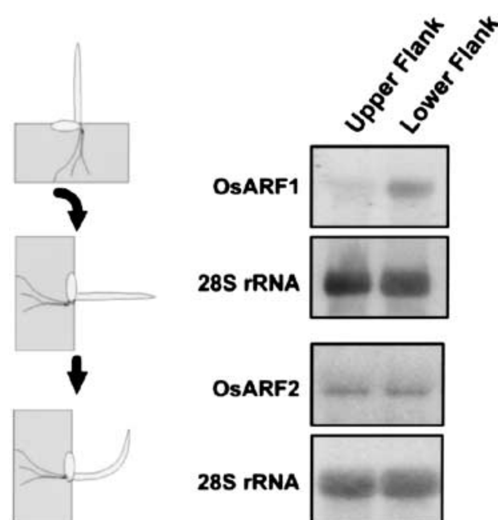

Figure 6. Expression of *OsARF1* and *OsARF2* mRNA after gravitropic stimulation. Rice seedlings were displaced from the vertical position by 90°. Upper and lower flanks of coleoptiles were harvested separately 180 min after the onset of this gravitropic stimulation and total RNA (15 μg) was used in northern blot experiments. Membranes were hybridized with *OsARF1* and *OsARF2* probes, and rehybridized with a rice 28S rRNA probe.

When 5-day old dark-grown rice seedlings were tilted by 90° from the vertical, the coleoptile began to bend upward within about 30 min, caused by an elevated elongation of the lower coleoptile flank. Within 3–4 h after the onset of gravitropic stimulation, the coleoptile tip returned to a vertical orientation (Wang and Nick, 1998). To assess the distribution of *OsARF1* mRNA during gravitropic bending, upper and lower flanks of gravitropically stimulated coleoptiles were harvested separately 15 and 180 min after the onset of gravitropic stimulation, and the *OsARF1* transcript levels compared in northern blot experiments. *OsARF1* mRNA accumulated in the (faster growing) lower flank of the coleoptile after 180 min, but was reduced in the (slower growing) upper flank (Figure 6). In contrast, no difference was detectable 15 min after the onset of gravitropic stimulation. Quantification of the *OsARF1* mRNA signal intensities on the northern blot showed a 1:2 ratio between the upper and the lower flank after 180 min of gravitropic stimulation (data not shown). In contrast to *OsARF1*, steady-state expression levels of *OsARF2* show no difference between the two flanks 180 min after the onset of gravitropic stimulation (Figure 6).

Discussion

In an approach to identify genes which products are involved in the regulation of plant growth, we applied fluorescent differential display (FDD) to the rice coleoptile, a model system for auxin-mediated elongation growth. *OsARF1*, an auxin response factor from rice, was identified to be differentially regulated by auxin and to be correlated in its mRNA expression with the growth response. Auxin response factors are transcription factors identified in *Arabidopsis* that regulate genes with AuxRE promotor elements, such as several early auxin induced genes (Ulmasov *et al.*, 1997a; Stowe-Evans *et al.*, 1998; Harper *et al.*, 2000). As the control of early auxin-responsive gene expression represents the earliest known step during auxin-induced gene expression, and an auxin-dependent expression of *ARF*s has not been described previously, we began to characterize *OsARF1*.

OsARF1*, a nuclear-localized putative repressor of early auxin-induced genes*

Isolation of the full-length *OsARF1* cDNA revealed the typical structure of an ARF: a N-terminal DNA-binding domain and two C-terminal domains (termed 'Box III' and 'Box IV'), which are implicated in protein dimerisation. In *Arabidopsis* it has been shown that the non-conserved middle region determines whether an ARF is an activator or repressor of *AuxRE* reporter genes in carrot protoplast cotransfection assays (Ulmasov *et al.*, 1999b). In this assay, *Arabidopsis* ARF1 with a middle region rich in proline, serine and threonine acts as a repressor, whereas other ARFs rich in glutamin act as activators. ARF1 is not only the closest known homologue to OsARF1, but is also sharing the feature of a P,S,T-rich middle region with OsARF1 (Figure 2). Thus, OsARF1 might act as a repressor of genes with AuxRE promotor elements.

Although the OsARF1 sequence indicates a function as a transcription factor, the OsARF1 amino acid sequence does not contain a typical nuclear localisation signal. We therefore tested the intracellular localization of OsARF1: transformation of tobacco protoplasts revealed a nuclear localization of the OsARF1:GFP fusion protein *in vivo*. This result supports the view that OsARF1 protein is a transcription factor localized to the nucleus.

Auxin-induced OsARF1 *mRNA expression and growth are closely correlated*

The expression pattern of *OsARF1* mRNA was closely correlated to the growth response of coleoptile segments, both in terms of auxin dose-response and time-course dependence. The optimal auxin concentration for the induction of elongation growth in rice coleoptiles is between 1 and 10 μM (Wang and Nick 1998). Highest levels of *OsARF1* mRNA were induced by auxin concentrations of between 1 and 3 μM (Figure 4). As soon as 15 min after the onset of auxin incubation, elevated steady-state *OsARF1* mRNA levels were detected (Figure 5C), correlating with the elongation of auxin-depleted coleoptile segments that started after a lag-phase of about 15 min (Figure 5B).

OsARF1 mRNA levels are not only regulated upon incubation with auxin, but also in coleoptiles of intact seedlings. Gravitropic stimulation of seedlings leads to differential growth of the upper and lower flank of the coleoptile, resulting in an upward bending of the coleoptile. In this situation, differential *OsARF1* mRNA levels between the upper and lower flanks of coleoptiles are observed, with higher *OsARF1* expression levels in the lower, faster-growing flank, and lower expression levels in the upper flank (Figure 6).

According to the Cholodny-Went theory (Cholodny, 1927; Went, 1929), the differential growth response during photo- and gravitropism is due to the lateral redistribution of auxin and the resulting different auxin concentrations in these tissues. The *OsARF1* transcript ratio of 1:2 between upper and lower flanks of gravitropically stimulated coleoptiles resembles the 1:2 ratio of auxin concentrations detected by different methods (Dolk, 1936; Gillespie and Thimann, 1963; Godbolé *et al.*, 2000).

In contrast, a second auxin response factor from rice, *OsARF2*, was not regulated by gravitropic stimulation (Figure 6), indicating that members of the ARF gene family in rice have specific functions.

Elevated levels of *OsARF1* transcript in tissues with an elevated growth rate (as observed in the lower flank of gravitropically stimulated coleoptiles) suggest that OsARF1 might positively regulate genes encoding products mediating the growth response. As the P,S,T-rich OsARF1 middle region suggests that OsARF1 acts as a repressor, the positive correlation of growth rate and high *OsARF1* transcript levels might be a consequence of OsARF1 negatively regulating a repressor. This repressor would control the expression of genes which products positively affect growth, such

as expansins. Candidates for this scenario would be Aux/IAA proteins, some of them having shown to be repressors of genes with AuxRE promotor elements (Ulmasov *et al.*, 1997b). Putative AuxREs have recently been identified in genes coding for proteins that might directly control the growth response, such as a maize gene coding for a K^+-channel implicated in coleoptile growth control (Philippar *et al.*, 1999, 2000) and an expansin gene from tomato (Caderas *et al.*, 2000).

Alternatively, fine-tuning of genes in growth control could be accomplished by OsARF1 acting in a negative feedback mechanism by repressing the expression of genes coding for positive regulators of growth. Furthermore, as it is known that ARFs bind AuxRE promotor elements as homo- or heterodimers (Ulmasov *et al.*, 1997, 1999b), differential expression of activating and repressing ARFs might lead to a different composition and activity of ARF dimers, which could be a fine-tuning mechanism for the regulated expression of auxin-induced genes.

OsARF1 *is an early auxin responsive gene*

We observed that upregulation of *OsARF1* steady-state mRNA levels was rapid and independent of *de novo* protein synthesis (Figure 5). Therefore, *OsARF1* classifies as a primary auxin responsive gene. This class of genes is supposed to be involved in the earliest steps of auxin signal transduction chains (Abel and Theologis, 1996). The protein synthesis inhibitor cycloheximide alone was also able to upregulate *OsARF1*, and the combined incubation in cycloheximide and auxin leads to a superinduction (Figure 6). Both effects have been observed for other primary auxin responsive genes previously (Franco *et al.*, 1990; Koshiba *et al.*, 1995), and are interpreted as cycloheximide influencing the synthesis of either a protein affecting mRNA stability, or a short-lived repressor protein.

The observed auxin-dependent regulation of *OsARF1* might be due to an AuxRE element in the promotor region of the *OsARF1* gene. As ARFs are responsible for regulating early auxin-induced genes via this element, OsARF1 might regulate its own transcription, which could provide the molecular basis of a feedback mechanism in auxin signaling. Such feedback mechanisms have been proposed to play a role in auxin-dependent processes, e.g. the formation of connected vascular strands: the canalization hypothesis proposes that higher local auxin concentrations lead to an enhanced capacity of cells to transport auxin (Sachs, 1981). Together with the polar distribution of auxin efflux carriers (Gälweiler *et al.*, 1998), this positive feedback mechanism could explain the development of interconnecting vascular strands during development. The auxin-regulated *OsARF1* might be, in a similar fashion, the basis of the tightly controlled differential growth response observed during the auxin-mediated gravitropic reaction of the coleoptile.

Neither auxin-dependent regulation nor auxin-dependent up-regulation independent of *de novo* protein synthesis has been reported previously for an *ARF*. Indeed, several ARFs from *Arabidopsis* have been reported to be unresponsive to auxin treatments (Ulmasov *et al.*, 1999a). Therefore our results indicate for the first time that another level of regulation in auxin-dependent gene expression – auxin-dependent regulation of *ARF* expression itself – exists. This could be one mechanism responsible for the highly specific expression, as observed for *ARF3* and *ARF5*, which control processes that depend on spatial auxin concentration gradients (Sessions *et al.*, 1997; Hardtke and Berleth, 1998). OsARF1, with its mRNA expression positively linked with elevated auxin-dependent growth might be a regulator of effector genes important for the elongation of plant cells. Tight transcriptional regulation of such effector genes, such as genes coding for expansins, might be accomplished by auxin-concentration dependent, as well as tissue-specific expression of ARFs and by dimerization of specific ARFs with other ARF and Aux/IAA proteins. Further elucidation of the downstream targets of OsARF1 will help to understand the regulation of plant growth by auxin-mediated gene expression.

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