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Phytohormone abscisic acid control RNA-dependent RNA polymerase 6 gene expression and post-transcriptional gene silencing in rice cells

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ABSTRACT

RNA-dependent RNA polymerase 6 (RDR6) catalyses dsRNA synthesis for post-transcriptional gene silencing (PTGS)-associated amplification and the generation of endogeneous siRNAs involved in developmental determinations or stress responses. The functional importance of RDR6 in PTGS led us to examine its connection to the cellular regulatory network by analyzing the hormonal responses of *RDR6* gene expression in a cultured cell system. Delivery of dsRNA, prepared *in vitro*, into cultured rice (*Oryza sativa* cv. Japonica Dongjin) cells successfully silenced the target isocitrate lyase (*ICL*) transcripts. Silencing was transient in the absence of abscisic acid (ABA), while it became persistent in the presence of ABA in growth medium. A transcription assay of the *OsRDR6* promoter showed that it was positively regulated by ABA. OsRDR6-dependent siRNA(*ICL*) generation was also significantly up-regulated by ABA. The results showed that, among the five rice *OsRDR* isogenes, only *OsRDR6* was responsible for the observed ABA-mediated amplification and silencing of *ICL* transcripts. We propose that ABA modulates PTGS through the transcriptional control of the *OsRDR6* gene.

INTRODUCTION

Post-transcriptional gene silencing (PTGS) involves degradation or translational repression of target RNA which binds small interfering (si)-RNA or microRNA by Watson–Crick base pairing. While microRNA is derived from genome-encoded precursor RNA with a stem-loop structure, siRNA is from dsRNA trigger, which can be formed either intracellularly or delivered exogeneously. dsRNA is cleaved into siRNAs of 20–25 nt in length by Dicer, a RNAaseIII-like enzyme (1). Down-regulation of target RNA by siRNA occurs within the RNA-induced silencing complex (RISC) containing *Argonaute* ribonucleoproteins (2). Primary siRNA and dsRNA PTGS trigger often undergo amplification processes through repeated polymerization to synthesize secondary dsRNA by RDR6 and subsequent dicing into secondary siRNAs by Dicer (3). Amplification involves a transitivity mechanism in which the secondary siRNAs being produced move across the original siRNA-target mRNA binding region to either the upstream or downstream direction in plants (4,5). The level of secondary siRNAs may be an important criterion for effective silencing or for cell-to-cell movement of siRNAs (4,6). It has recently been suggested that the fate of target RNA, either used for cleavage or for polymerization, depends on the kinds of *Argonaute* proteins involved in the guidance of siRNA and target RNA into the RISC (2,7).

RNA-dependent RNA polymerase (RDR) is known to be responsible for the synthesis of dsRNA on ssRNA substrates in either a primer-dependent or primer-independent manner. Such synthesis has been reported in *C. elegance*, *Dictyostelium* and plants, but not in *Drosophila* and mammals (8). There are six RDR isogenes in *Arabidopsis—AtRDR1, AtRDR2, AtRDR3a, AtRDR3b, AtRDR3c* and *AtRDR6*, while rice has five homologues—*OsRDR1, OsRDR2, OsRDR3a, OsRDR3b* and *OsRDR*6 (8,9). Among these, AtRDR2 is known to participate in transcriptional gene silencing by forming dsRNA from which chromatin-targeting siRNAs are produced (10,11), while AtRDR6 participates in the synthesis of dsRNA from various RNA substrates that are derived from transgene, viral replication, convergent transcription or microRNA-cleaved mRNA (8). It is also suggested that RDR1 plays a role in resistance to herbivore attack in tobacco (12). No function has been assigned for the other RDR isozymes. Over-expression or multi-copies of transgenes tend to generate aberrant RNAs that often

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contain truncated RNA structures lacking a 3′-polyA tail and/or 5′-cap (13,14). These aberrant RNAs are reported to preferentially recruit RDR6 for dsRNA synthesis. RDR6 prefers to initiate polymerization at the 3′-end in the absence of primer in plants (15–17), although both primed- and unprimed-polymerizations have been demonstrated *in vitro*. Consistent with these notions, endogeneous mature mRNAs have not been demonstrated to be a target for RDR6-mediated amplification in plants (5).

RDR6 also plays an important role in the control of development progresses or stress resistance by synthesizing dsRNA, which are diced into endogeneous siRNAs such as *trans*-acting (tasiRNA) (18,19) and natural siRNAs (nat-siRNA) (20), respectively. tasiRNA is derived from the non-coding TAS transcript, which is targeted by a specific microRNA. MicroRNA390 is known to cleave the TAS3 transcript, and the cleavage product is stabilized and polymerized into dsRNA through the activity of RDR6 and Suppressor of Gene Silencing3 (SGS3) (18,19). DCL4, a Dicer-like ribonuclease, dices the dsRNA into tasiRNAs in a phased manner, and these tasiRNAs bind target mRNA for cleavage (21,22). It has also been shown that, as exemplified in *Arabidopsis* TAS3 RNA, microRNA390-cleaved RNA is a preferred target for RDR6 (23). siRNA–AGO ribonucleoprotein complex recruits RDR6 to initiate primer-dependent polymerization on the microRNA390-cleaved TAS3 RNA substrate in *Arabidopsis*. One of the tasiRNA targets is an auxin responsive factor3 (*ARF3*, also called *ETTIN*) transcript, which is a mediator of auxin signaling involved in cellular differentiation, such as leaf polarity (15,24,25). Because RDR6 is a critical component in the control of developmental processes, it seems logical that its expressional regulation is linked to an internal signaling network. This study demonstrates that ABA up-regulates transcription of the rice *OsRDR6* gene, indicating that *OsRDR6* gene expression is a target of hormonal control.

MATERIALS AND METHODS

Maintenance of rice suspension cultures and plants

Rice cell culture originated from the ovules (germinal vesicles) of *Oryza sativa* Japonica. cv. Dongjin, and it was maintained at 25°C with constant agitation in darkness by weekly subculturing. The culture medium contained basal N6 medium components (26) and vitamins, 0.2 mg/l of kinetin, 2 mg/l of 2,4-D and 3% sucrose (pH 5.8). For the acetate culture, sucrose was replaced with 10 mM potassium acetate (pH 5.8).

Formation of dsRNA/cationic peptide complex and its delivery

About 100 ng of POA (polyarginine 12-mer) (synthesized by Peptron, Inc. Korea) were mixed with 100 ng of dsRNA to form a delivery-effective complex in which dsRNA is encased within the POA peptide shell. To deliver less amounts of dsRNA, amounts of POA were accordingly lowered. This complex was confirmed by agarose gel electrophoresis in which dsRNA was not stained with ethidium bromide. The complex was added to 0.5 g of fresh cells and was incubated for 1 h at 25°C in a total volume of 300 µl.

RT-PCR

Total RNA was prepared from the samples using TRI reagent (Molecular Probes Diagnostics, USA) according to the manufacturer's instructions. The first-strand cDNA was synthesized from 2 µg of total RNA in a volume of 20 µl using a evertAid_First Strand cDNA Synthesis Kit (Fermentas, Lithuania). One microliter of the first-strand solution was used for the PCR. The annealing temperature was 55°C for OsRDR6 and ICL, 63°C for OsRDR3a, and 50°C for actin, and 30 cycles of PCR were performed. The following gene-specific primers were used to detect the gene expression level; ICL-FP (5′-CAGCTCAAGAC CTTCTCTGA-3′) and ICL-RP (5′-CTCTGGATCCT CTCCACGTA-3′); RDR6-FP (5′-TCACTTAGCTGA GCTAGCAG-3′) and RDR6-RP (5′-TCGGAGATGT ATGCTGCAAG-3′); *OsRDR3a*-FP (5′-TACGGCAGC TGATAGCTGGT-3′) and *OsRDR3a*-RP (5′-CTGGT AGATCACGCAGGCTT-3′). The rice actin gene (forward, 5′-TCCATCTTGGCATCTCTCAG-3′; reverse, 5′-GTACCCGCATCAGGCATCTG-3′) was used as an internal control.

Detection of siRNA by RNase protection assay

In order to enrich small size-RNA, 50 µg total RNA was mixed with PEG8000 (5%) and NaCl (0.5 M), then incubated on ice for 30 min and centrifuged at 13 000*g* for 15 min. Small size-RNA was enriched in the supernatant. By adding 10 µg of yeast tRNA and three volumes of EtOH to the supernatant and incubating for 30 min at –70°C, small size-RNA was precipitated and used for the RNase protection assay. The small size-RNA was hybridized to α-^{32}P-UTP-labeled sense RNA (internal region of *ICL*) overnight at 45°C, and the unhybridized ssRNA portions were digested by RNase A. After the debris was washed out, the hybridized fraction was precipitated with 1 µl of yeast tRNA (10 mg/ml) and three volumes of EtOH. The pellet was dissolved in 10 µl of formaldehyde–RNA dye and loaded into a 15% denaturing PAGE gel, which was exposed to X-ray film.

Transcription assay

Arabidopsis protoplasts were isolated from *Arabidopsis* 3-week-old seedlings. The whole plant was soaked in 25 ml enzyme solution (1% cellulase R-10, 0.25% macerozyme R-10, 400 mM Mannitol, 8 mM CeCls, 5 mM Mes with pH 5.6 KOH) at 22–25°C for 2–4 h. The protoplasts were carefully filtered with 100 mm mesh to prevent damage and mixed with a final volume of 25 ml W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 1.5 mM Mes with pH 5.6 KOH). Following centrifugation at 40*g* for 5 min at 25°C, the supernatant was removed by pipette and the remaining pellet was suspended in W5 solution. After checking the protoplasts under an optical microscope, the mixture was gently loaded on top of a 21% sucrose solution and enriched by centrifugation at 40*g* for 5 min at 25°C. The enriched

protoplasts were removed from the middle of the mixture with a pipet, resuspended in 20 ml W5 solution, and stored for 2–4 h at 4°C. The enriched pellet was suspended with MaMg solution (400 mM mannitol, 15 mM $MgCl_2$, 5 mM MES with pH 5.6). Next, plasmid DNA was added, and the mixture was immediately suspended in PEG solution (40% PEG4000, 200 mM Mannitol, 100 mM $CaCl_2$). Then, after flow-through of the same volume of MaMg solution, the mixture was incubated for 30 min at 25°C. The transformed mixture was washed with W5 solution and centrifuged at 40*g* for 5 min at 25°C, after which the pellet was resuspended in 2 ml of W5 solution.

RDR activity assay

Harvested rice suspension cells were ground by a mortar and pestle in 0.7 volumes of grinding buffer (110 mM KOAc, 20 mM HEPES–KOH at pH 7.3, 1 mM MgOAc, 1 mM DTT, 3 mM EGTA, 2 mM $CaCl_2$). After centrifugation of the homogenate, the supernatant was used for the RDR activity assay. For primer-independent polymerization, the reaction was initiated by adding 50 μg of each cell extract to a reaction mixture containing 5 μg of sense-stranded template RNA (ICL), 2 μl polymerization buffer (50 mM HEPES–KOH at pH 7.8, 20 mM NH_4OAc, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Triton X-100), 0.5 mM ATP, 100 μM CTP, 100 μM GTP, 20 μM UTP, 25 μCi α-^{32}P-UTP and 0.2 U/μl RNsin (Ambion) in a total volume of 20 μl. After incubating the mixtures at 25°C for 3 h, the reaction was stopped by vortexing with an equal volume of phenol/chloroform/isoamylalcohol. After centrifugation, the supernatant was precipitated with 1 μl of yeast tRNA (10 mg/ml) and three volumes of EtOH. The pellet was dissolved in 10 μl of formaldehyde–RNA dye and loaded into an 8% denaturing PAGE gel. For siRNA-dependent activity, template RNA, which was annealed to α-^{32}P-UTP labeled siRNA (corresponding to the region 150 bp from the 5′-end of the template) at room temperature for 10 min, was used along with the other reaction components (without α-^{32}P-UTP) to initiate the reaction.

Primer extension

Primer extension analysis was carried out as described previously (27), with some modifications. Total RNA (10 μg) was used for annealing to the 5′-end-labeled primer 1 (5′-TGTCCATGCCCAAACTCCAA-3′) and 5′-end-labeled primer 2 (5′-AAAGCAGCACGGCCATGA AG-3′) at 35°C for 16 h and for subsequent extension using SuperScript II (Invitrogen, CA) at 42°C for 2 h. Samples were separated by 6% denaturing PAGE gel and autoradiographed.

RESULTS AND DISCUSSION

To establish an experimental PTGS system in cultured rice cells, we attempted to silence a gene for isocitrate lyase (*ICL*), which, as a glyoxylate cycle enzyme, is induced by acetate (Figure 1A). Plant cells are known to operate a glyoxylate cycle to circumvent carbon loss during the citric acid cycle in the presence of acetate as a sole carbon source (28). Acetate-induced ICL mRNA was subjected to PTGS by delivering dsRNA covering the 0.5 kb *ICL* coding region, which is synthesized *in vitro* (hereafter designated as dsICL). dsICL was delivered into cells with a polyarginine oligopeptide (POA) carrier, which protects nucleic acids within its shell as a result of positive charges on its surface (29,30). As shown in lane 8 and thereafter in Figure 1B, dsICL appeared to be protected by the POA shell and ready for delivery, as evidenced by negligible ethidium bromide staining in the mobility retardation assay. The ratio of POA/dsICL applied in lane 8 was used for the rest of the studies.

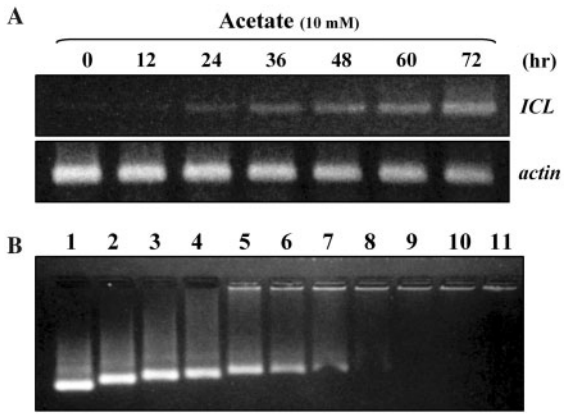

Figure 1. Formation of polyarginine(POA)/dsICL complex for silencing of *ICL*. (**A**) 10 mM acetate in medium gradually up-regulated the level of *ICL* transcripts during the 72 h period examined. (**B**) Formation of the dsRNA(*ICL*)/POA complex. To evaluate optimum complex formation, various concentrations of POA were mixed with a fixed concentration of dsRNA (lane 1, 100 ng of dsRNA only; lanes 2–11, 12.5, 25, 37.5, 50, 62.5, 75, 87.5, 100, 112.5 and 125 ng of POA mixed with 100 ng of dsRNA, respectively). As the POA concentration increased, dsRNA mobility was gradually retarded. The POA concentration in lane 8 was chosen as the most effective delivery-complex.

ICL mRNA was silenced when 10 μg of dsICL was delivered into cells growing in acetate (Figure 2A). The *ICL* mRNA level was down-regulated transiently around 12 h following dsICL delivery. After 48 h, *ICL* mRNA appeared to recover to its normal level, indicating that silencing was not persistent. The appearance of siRNA was monitored to investigate the progress of PTGS (Figure 2B). Enriched siRNAs were hybridized to α-^{32}P-UTP-labeled sense RNA corresponding to the region used for dsICL preparation, and siRNAs were detected after enzymatic degradation of the unhybridized sense RNA portion. In agreement with the transient decrease of *ICL* mRNA, siRNA appeared transiently at 12 h (Figure 2B). The *OsRDR6* transcript level was down-regulated in the presence of acetate (Figure 3A), strongly suggesting that RDR6-mediated amplification of dsRNAs and siRNAs did not occur in cells grown in acetate. It is very likely that primary siRNAs, derived from the delivered dsICL trigger, were used to target *ICL* mRNA for cleavage to complete the single round of silencing in the absence of activity of OsRDR6. This PTGS system designed for silencing of *ICL* transcript was used to investigate expressional control of *OsRDR6* and the accompanying PTGS.

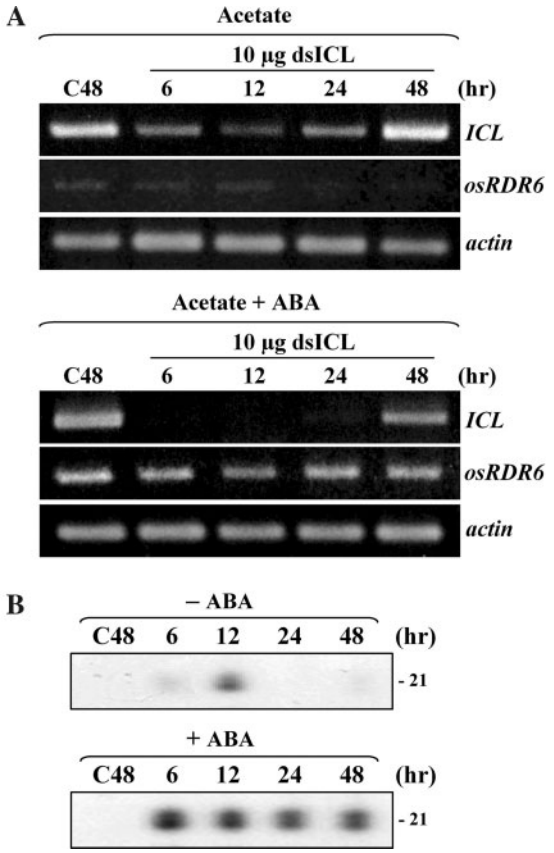

Figure 2. PTGS of ICL transcripts and generation of siRNA. (**A**) Silencing of *ICL* transcripts in the absence (upper) and presence (bottom) of ABA. Following delivery of 10 μg of dsICL into cells pre-cultured for 48 h in acetate, the levels of ICL transcripts were monitored by RT–PCR. (**B**) Detection of siRNA in cells grown in the absence (upper) and presence (bottom) of ABA. Following the delivery of dsICL, appearance of siRNA was monitored. In dsICL-free control cells, siRNA did not appear at 48 h following the transfer into ABA-containing medium (C48).

Initially, the presence of an ABA-responsive DNA *cis*-element (ACGTG) at the –680 region of *OsRDR6* promoter prompted us to examine whether ABA initiated amplification to allow persistent PTGS. RT–PCR analyses showed that the *OsRDR6* transcript level was up-regulated in the presence of ABA (Figure 3B). Northern analyses also showed that ABA up-regulated *OsRDR6* expression in cells grown either in acetate or sucrose (Figure 3C), indicating that ABA induction was not limited to cells grown in acetate. In line with this observation, the silencing of *ICL* transcripts was significantly enhanced in the presence of ABA in cells grown in acetate (Figure 2A). It was shown that *ICL* transcripts were not detected up to 24 h, although they appeared again, at a lower level, at 48 h. siRNAs were detected throughout the examination (Figure 2B), indicating that ABA induced efficient PTGS of *ICL* transcripts through the persistent generation of siRNA. Re-appearance of the *ICL* transcript at 48 h (Figure 2A) was likely because PTGS of *ICL* mRNA was less effective at 48 h. Sufficient amounts of target mRNAs are probably an important factor in the continuous activity of OsRDR6. If secondary siRNAs are defined as

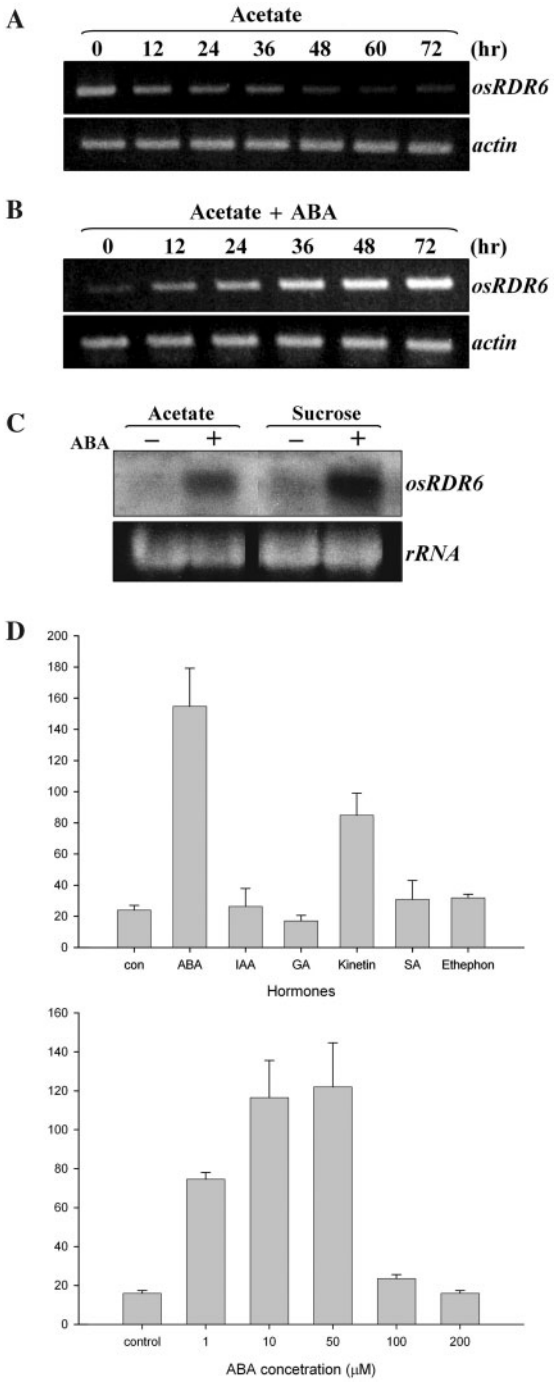

Figure 3. ABA regulation of *OsRDR6* gene expression. RT–PCR analyses of changes of *OsRDR6* transcript level following the transfer into acetate medium without ABA (**A**) and with ABA (**B**). (**C**) Northern analyses of ABA up-regulation of OsRDR6 gene expression in cells grown in acetate (10 mM) or sucrose (3%). (**D**) Transcription assays of *OsRDR6* promoter (1.2 kb). A promoter:luciferase construct was transfected into *Arabidopsis* protoplasts, and they were incubated with different plant hormones (upper), and with different concentrations of ABA (bottom). Variations of the three independent assays are shown.

'siRNAs synthesized from RDR6-generated dsRNA', the siRNAs produced in the presence of ABA should be considered secondary siRNAs. Although the nature of these siRNAs was not analyzed further, we conclude that

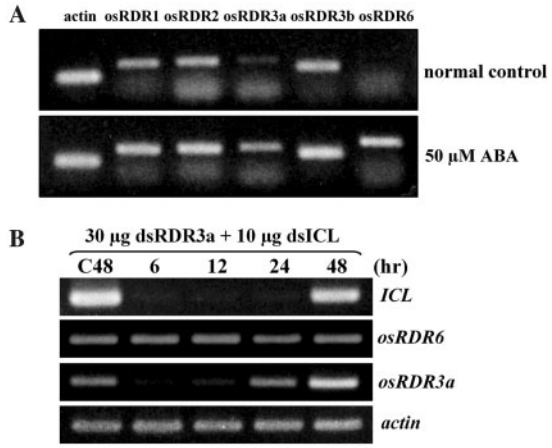

Figure 4. ABA responses of rice RDR homologues. (**A**) RT–PCR analysis of ABA responsiveness of rice *RDR* homologues. (**B**) Silencing of *OsRDR3a transcripts* by delivering dsOsRDR3a, together with dsICL, into cells growing in acetate in the presence of ABA. Following the delivery of dsOsRDR3a, levels of *ICL*, *OsRDR3a* and *OsRDR6* transcripts were evaluated by RT–PCR.

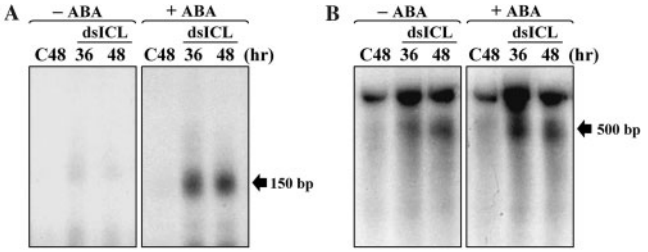

Figure 5. Characterization of RNA polymerization, performed with cell extracts in the presence (**A**) and absence (**B**) of siRNA primers. Sense-stranded RNA corresponding to the internal ICL region (0.5 kb) was used as a template for polymerization in the polymerization mixture containing α-^{32}P-UTP. In the presence of ABA (right columns), both primed- and unprimed-polymerization appeared to be induced (see 150 and 500 bp expected polymerization products, respectively), while only unprimed polymerization operated in dsICL-delivered cells in the absence of ABA (B, left).

these secondary siRNAs were derived from secondary dsRNAs synthesized by ABA-induced activity of OsRDR6. To further confirm ABA control, transcription assays were performed to examine the promoter activity of the *OsRDR6* gene. A plasmid vector carrying the 1.2 kb region of the promoter region, which is fused to the luciferase gene, was transfected into *Arabidopsis* protoplasts to monitor transcriptional activity (Figure 3D). The results showed that *OsRDR6* gene was transcriptionally up-regulated by ABA. The optimum concentration of ABA was about 50 μM. Of the other hormones, only kinetin appeared to be somewhat effective.

To investigate whether the other *OsRDR* isogenes, in addition to *OsRDR6*, are also involved in the amplification of secondary dsICL and siRNAs, the rice *RDR* homologues were examined for their responses to ABA (Figure 4A). Among the five rice *OsRDR* homologues, levels of *OsRDR6* and *OsRDR3a* transcripts were significantly up-regulated by ABA, suggesting that OsRDR3a may also participate in amplification in the presence of ABA. To examine the participation of *OsRDR3a* in PTGS of *ICL* transcripts, dsRNA spanning the *OsRDR3a* coding region (dsOsRDR3a) was delivered to silence *OsRDR3a* transcripts (Figure 4B). Delivered dsOsRDR3a silenced *OsRDR3* transcripts efficiently, without silencing *OsRDR6* transcripts. *OsRDR6*-mediated silencing of *ICL* transcripts in the presence of ABA appeared not to be affected by the silencing of *OsRDR3a* transcripts. These results strongly suggested that ABA-induced amplification was exclusively due to the activity of OsRDR6. Although *OsRDR3a* expression was responsive to ABA, its does not appear to participate in the amplification of PTGS. The function of OsRDR3a is yet to be characterized.

To further characterize ABA up-regulation of *OsRDR6* expression, we analyzed RNA polymerization activity (Figure 5). With cell extracts prepared from acetate-grown cells, polymerization of an antisense cRNA strand on the *ICL* internal sense RNA template (0.5 kb) was examined either with siRNA(*ICL*) as a primer or without primer. Primer-dependent polymerization was initiated by adding the annealed siRNA(α-^{32}P-labeled)/sense RNA template complex to the extracts, with an expected product of 150 bp (Figure 5A). Primer-dependent activity was clearly induced in cells treated with dsICL and ABA, while it was negligible in cells treated only with dsICL without ABA or in cells treated only with ABA without dsICL. Primer-independent polymerization was similarly up-regulated by ABA in these cells, except that it was also active in cells treated only with dsICL without ABA (Figure 5B). Primer-independent polymerization operated predominantly in the absence of ABA, suggesting that it constitutively operates in the presence of dsICL regardless of the presence of ABA. Taken together, we conclude that ABA activated OsRDR6-mediated polymerization following the transcriptional up-regulation of the *OsRDR6* gene.

Although both primer-dependent and primer-independent activities were active in these cell extracts, the actual mode of polymerization *in vivo* is not understood. Primer-independent polymerization is known to be predominant in plants, as aberrant RNAs are usually used as substrates for RDR6 (16). Mature endogeneous mRNAs have never been demonstrated as a substrate for amplification. It is probable that, as an aberrant RNA, delivered dsICL was a favorable substrate for OsRDR6-mediated polymerization to amplify the secondary dsRNAs and secondary siRNAs to degrade large amounts of acetate-inducible *ICL* mRNA. It remains to be characterized if *ICL* mRNA is used for amplification by primer-dependent polymerization in the presence of ABA.

Knowing that RDR6 is also an important component in the generation of endogeneous siRNAs, such as tasiRNAs and nat-siRNAs in *Arabidopsis*, we wanted to see if this phenomenon also operated in cultured rice cells in relation to ABA. To examine if ABA is also involved in the up-regulation of *OsRDR6* expression to enhance the efficiency of tasiRNA generation, we chose a pathway in which microRNA390-cleaved TAS3 precursor is cleaved into tasiRNAs. RDR6-mediated tasiRNA synthesis, in conjunction with DCL4 and AGO1/7, is responsible for several aspects of plant development in *Arabidopsis* (31).

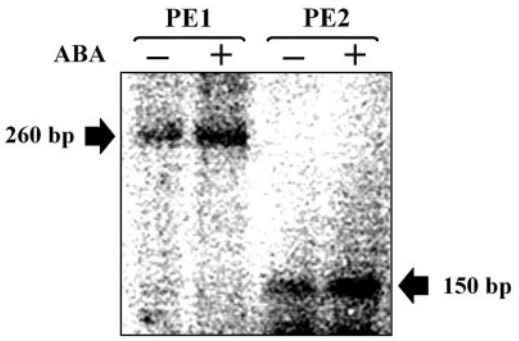

Figure 6. ABA regulation of tasiRNA-ARF generation. The tasiRNA-ARF level was measured by monitoring cleavage of *ARF3* mRNA by primer extension (PE) assays. Two primers were designed to extend to the predicted cleavage sites and generate products of 260 bp (PE1) and 150 bp (PE2) (arrows).

A group of these tasiRNAs is known to target *ARF3* mRNA for cleavage in *Arabidopsis* (designated tasiRNA-ARF). However, we were not able to detect tasiRNA-ARF in cultured rice cells treated with ABA, probably because the level of tasiRNA-ARF was too low to detect in these cultured cells even in the presence of ABA. On the assumption that tasiRNA-ARF synthesis was up-regulated by ABA, levels of the cleavage products of *ARF3* mRNA were alternatively analyzed for the ABA response, since cleavage of *ARF3* mRNA is derived from its binding to tasiRNA-ARF. The suggested cleavage site for tasiRNA-ARF within the *ARF3* mRNA in *Arabidopsis* was also confirmed in rice cells (Figure 6), indicating that this endogeneous pathway is conserved among these plants. Primer extension assays were performed to detect the primer-extended cleavage fragments, which spans from the primer-binding site to the expected cleavage site (Figure 6). Two different primers were used to produce the expected sizes of 260 bp (PE1) and 150 bp (PE2). The levels of the two predicted cleavage fragments were significantly higher in ABA-treated cells, strongly suggesting that ABA-induction of the *OsRDR6* gene was responsible for enhanced synthesis of tasiRNA-ARF and cleavage of *ARF3* mRNA.

This study clearly demonstrates that transcription of the *OsRDR6* gene is positively regulated by ABA. Cleavage of target transcripts by siRNAs, which are derived from OsRDR6-generated dsRNA, was accordingly up-regulated by ABA. This study provides a mechanistic model in which PTGS duration is extended by up-regulating *RDR6* gene expression. QDE-1, a RDR6 equivalent in *Neurospora crassa*, is a rate-limiting factor for QDE-1-mediated PTGS, and the efficiency and persistence of PTGS depends on the amount of cellular QDE-1 protein (32). Since ABA signaling is widely interconnected with other hormones and internal regulatory networks, the results demonstrated in this study suggest that PTGS is closely associated with a cellular signaling network at least via OsRDR6. Although other PTGS components were not investigated, transcriptional control of the *OsRDR6* gene appears to be a major target for hormonal control. Hormones may participate in gene silencing by regulating the HYL1 protein, which, as a dsRNA-binding protein, is known to affect accumulation of microRNA (33). Mutation in the *hyl1* gene disrupts plant sensitivity to ABA, auxin and cytokinin, implying that the biogenesis of microRNA is linked to hormonal regulation. ABA is known to control gene expression of several RNA binding proteins, suggesting that ABA is involved in many aspects of RNA processing, such as pre-mRNA splicing, 5-capping, 3′-polyA tailing, or export from the nucleus (34). It was also shown that ABA directly binds a nuclear RNA binding protein FCA to control autonomous floral pathway (35). As this study indicates, ABA is also involved in determining the cellular level of secondary siRNAs via transcriptional up-regulation of *OsRDR6* gene. We conclude that PTGS involving RDR6-mediated RNA polymerization is closely associated with a hormonal signaling network.

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Conflict of interest statement. None declared.

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