DNA Double-Strand Breaks Serve as a Major Factor for the Expression of *Arabidopsis Argonaute 2*

Sungbeom Lee[1,2,†,*], Moon-Soo Chung[1,†], Gun Woong Lee[1] and Byung Yeoup Chung[1,*]

[1]*Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, 29, Geumgu-gil, Jeongeup-si, Jeollabuk-do 56212, Republic of Korea*
[2]*Department of Radiation Biotechnology and Applied Radioisotope Science, University of Science and Technology, 217, Gajeong-ro, Yuseong-gu, Daejeon 34113, Republic of Korea*

Abstract - Argonaute 2 (AtAGO2) is a well characterized effector protein in *Arabidopsis* for its functionalities associated with DNA double-strand break (DSB)-induced small RNAs (diRNAs) and for its inducible expression upon γ-irradiation. However, its transcriptional regulation depending on the recovery time after the irradiation and on the specific response to DSBs has been poorly understood. We analyzed the 1,313 bp promoter sequence of the *AtAGO2* gene (*1.3kb$_{pro}$*) to characterize the transcriptional regulation of *AtAGO2* at various recovery times after γ-irradiation. A stable transformant harboring *1.3kb$_{pro}$* fused with *GUS* gene showed that the *AtAGO2* is highly expressed in response to γ-irradiation, after which the expression of the gene is gradually decreased until 5 days of DNA damage recovery. We also confirm that the *AtAGO2* expression patterns are similar to that of γ-irradiation after the treatments of radiomimetic genotoxins (bleomycin and zeocin). However, methyl methanesulfonate and mitomycin C, which are associated with the inhibition of DNA replication, do not induce the expression of the *AtAGO2*, suggesting that the expression of the *AtAGO2* is closely related with DNA DSBs rather than DNA replication.

Key words : Genotoxins, Methyl methanesulfonate, Mitomycin C, Gamma-rays, *Arabidopsis thaliana*

INTRODUCTION

Argonaute (AGO) proteins are important components of various molecular mechanisms of RNA silencing and DNA double-strand breaks (DSBs) repair system (Mallory and Vaucheret 2010; Parker 2010; Wei *et al*. 2012). In *Arabidopsis thaliana*, there are 10 AGO genes. The 10 AGO proteins are categorized into three clades based on sequence similarity: clade I, AGO1, AGO5, and AGO10; clade II, AGO2, AGO3, and AGO7; and clade III AGO4, AGO6, AGO8, and AGO9 (Morel *et al*. 2002; Vaucheret 2008). The AGO1 protein is involved in plant development and has antiviral roles (Zhang *et al*. 2006; Bohmert *et al*. 1998). In addition, AGO1, AGO2, AGO4, AGO5, AGO7, and AGO10 have been reported to have antiviral activities against *Turnip mosaic virus*, *Turnip crinkle virus*, and *Cauliflower mosaic virus* (Qu *et al*. 2008; Raja *et al*. 2014; Garcia-Ruiz *et al*. 2015). The expression of *Arabidopsis AGO2* (*AtAGO2*) was strongly induced by γ-irradiation in both mRNA and protein levels, and that the *ago2* mutant showed not only reduced accumulation of DSB-induced small RNAs (diRNAs), but also reduced DSB repair rate compared to wild-type (Wei *et al*. 2012). Moreover, AtAGO2 regulates the innate immunity upon the infection with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*), which triggers host DNA DSBs in *Arabidopsis* (Zhang *et al*. 2011; Song and

† These authors contributed equally to this work.
* Co-corresponding author: Byung Yeoup Chung, Tel. +82-63-570-3331, Fax. +82-63-570-3391, E-mail. bychung@kaeri.re.kr
Sungbeom Lee, Tel. +82-63-570-3384, Fax. +82-63-570-3391, E-mail. sungbeom@kaeri.re.kr

Table 1. Primers used in the construction of the *AtAGO2* promoter deletion mutants and RT-PCR analysis

Construct	Sequence (5′ to 3′)	
	Forward	Reverse
1.3kb$_{pro}$	CACCTATGACTTCGATGATGCTAAATCACAATTTGGTATTG	CTCCATTGGATCTGATCGGGAAACACTGAG
AtAGO2	AGGGATAGGGACAAGCAAACCG	CAGCGAGTGAAAGTGAAGCACA
GUS	CAGGAGAAACTGCATCAGCC	GATCCCTTTCAAGTTACCGC
AtACT2	ATTCAGATGCCCAGAAGTCTTGTT	GAAACATTTTCTGTGAACGATTCCT

Bent 2014). In addition to γ-irradiation, effects of genotoxins on the damage of DNA or inhibition of DNA replication have been reported. For example, bleomycin and zeocin, which are known as radiomimetic genotoxins, have also been known to induce DNA DSBs (Chankova *et al*. 2007). On the contrary, an aziridine-containing natural product mitomycin C (MMC) and an alkylating agent methyl methanesulfonate (MMS) are believed to be associated with the inhibition DNA replication (Menke *et al*. 2001).

Given that the expression of the *AtAGO2* is inducible upon γ-irradiation, information on the recovery time-dependent regulation of the *AtAGO2* after the irradiation has been limited. We characterized stable transgenic *Arabidopsis* plants, which harbor the full-length promoter of *AtAGO2* (1,313 bp) fused with GUS, after the treatments of γ-rays or four genotoxins. Here, we present the comparative transcript levels between *AtAGO2* and *GUS* at various recovery times after γ-irradiation, together with histochemical assay of GUS protein. We also show the gene expressions in response to MMC and MMS treatments to characterize the DSB-specific response of the *AtAGO2*.

MATERIALS AND METHODS

1. Plant treatments

An *Arabidopsis* (Columbia-0) transformant was grown at 22°C under 16 h light/8 h dark cycles, 100-150 μmol m^{-2} s^{-1} light intensity, and 60-70% relative humidity. For ionizing radiation, two-week-old transgenic seedlings were irradiated at 200 Gy (50 Gy h^{-1} for 4 h) using a ^{60}Co source (IR-222; MDS Nordion Inc., Kanata, Canada) (Kim *et al*. 2010). For bleomycin (BLM; Sigma, MO, USA) and zeocin (ZEO; Invitrogen, CA, USA) treatments, transgenic plants were incubated with 33 μM of BLM and 175 μM of ZEO for 1 h, respectively. The plants were subsequently transferred to a half-strength MS media and collected at indicated times after recovery. For mitomycin C (MMC; Sigma, MO, USA) and methyl methanesulfonate (MMS; Sigma, MO, USA) treatments, transgenic plants were incubated with 0.5 mM and 5 mM for 2 h, respectively.

2. Preparation of an *AtAGO2* promoter-*GUS* construct and *Agrobacterium*-mediated plant transformation

Genomic DNAs were isolated from seedlings using the Exgene™ Plant SV mini Kit (GeneAll, Seoul, Republic of Korea), following the manufacturer's protocol. The 1,313 bp *AtAGO2* promoter was amplified from the genomic DNA by using a proof-reading polymerase with the primers harboring the "CACC" sequence in forward primers for the properly oriented DNA recombination (Table 1). The resulting amplicon was cloned into the pENTR™/D-TOPO® vector (Invitrogen, CA, USA), and then integrated into the pKGWFS7 vector to fuse with β-glucuronidase (*GUS*) gene (Karimi *et al*. 2002) using the Gateway™ LR Clonase™ II Enzyme Mix (Invitrogen, CA, USA). The final construct was introduced into *Arabidopsis* by *Agrobacterium tumefaciens* (strain GV3101)-mediated *in planta* vacuum infiltration (Bechtold and Pelletier 1998).

3. Histochemical GUS staining analysis

Histochemical staining for GUS activity in the transgenic plants was performed as previously described (Jefferson *et al*. 1987). After the treatments, whole plants were immersed in 2.4 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid solution (GoldBio, MO, USA) containing 100 mM sodium phosphate, pH 7.0, 10 mM Na_2EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% (v/v) Triton X-100, and 20% (v/v) methanol, and incubated at 37°C for 24 h. Chlorophylls were removed from the plant tissues by immersion in absolute ethanol at 55-60°C.

4. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from plant leaf tissues using an RNeasy® Plant Mini Kit (Qiagen, CA, USA), following the manufacturer's recommendation. Total RNA was quantified with Nano-Drop (Thermo Scientific, DE, USA) and reverse-transcribed to complementary DNA using a GoScript™ Reverse Transcription System (Promega, WI, USA). The PCR amplification (28-30 cycles of 95°C for 40 s/51°C for 30 s/72°C for 1 min) was performed using the gene-specific primers (Table 1).

RESULTS

1. The expression pattern of *AtAGO2* in response to γ-irradiation at various recovery times

We employed transgenic *Arabidopsis* plants containing an *AtAGO2* promoter-GUS construct (*1.3kb$_{pro}$-GUS*) to monitor the expression pattern of *AtAGO2* gene in response to γ-irradiation at various recovery times. Two-week-old transgenic plants were exposed to 200 Gy of γ-irradiation for 4 h, and GUS expression at various recovery days after γ-irradiation (RAI) were observed using a histochemical staining and transcript analysis, compared in parallel with the transcript accumulation patterns of endogenous *AtAGO2* gene (Fig. 1). Histochemical analysis showed that the GUS expression was highly induced at 1 RAI and 2 RAI, and the expression was dramatically decreased over 3 RAI (Fig. 1B). No GUS accumulation was observed at 5 RAI (Fig. 1B). These results were comparable with the transcript levels of endogenous *AtAGO2* and *GUS* genes. The expressions of the *AtAGO2* and the *GUS* transcripts were immediately induced in response to γ-irradiation. However, the expressions were gradually decreased over 1 RAI, continuously maintaining higher expression levels than that of the control during 5 d of recovery time (Fig. 1C).

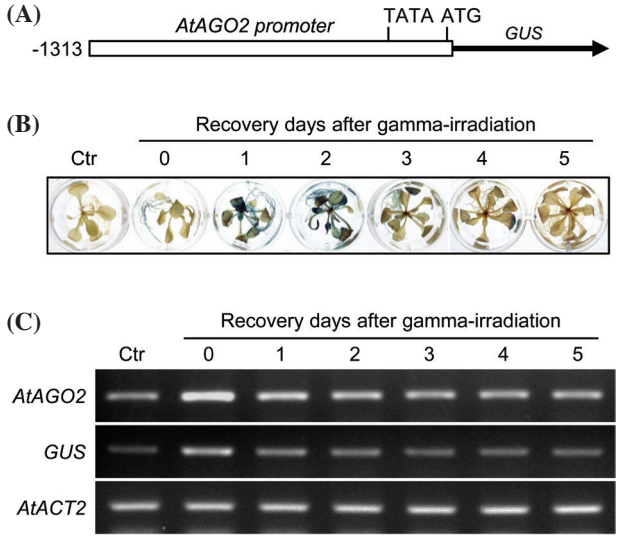

Fig. 1. Response of *AtAGO2* promoter at various recovery days after γ-irradiation (RAI). Two-week-old seedlings of the *1.3kb$_{pro}$-GUS* overexpression mutant were irradiated at 200 Gy of γ-rays, followed by 1, 2, 3, 4, and 5 d of incubation at room temperature. (A) Schematic representation of the *AtAGO2* full-length promoter construct, fused with *GUS* gene (*1.3kb$_{pro}$-GUS*), (B) Histochemical GUS staining at the indicated RAI, and (C) Steady-state transcript levels of *AtAGO2* and *GUS* at the indicated RAI. An *AtACT2* is used as an endogenous control gene. Ctr, no treatment.

2. Recovery time-dependent response of *AtAGO2* promoter after the treatments of radiomimetic genotoxins

We considered DNA DSB-inducing genotoxic agents, bleomycin and zeocin, to mimic the effects of γ-rays on DNA molecules (Menke *et al*. 2001; Chankova *et al*. 2007; Kozak *et al*. 2009). The two-week-old *1.3kb$_{pro}$-GUS Arabidopsis* plants were challenged with 33 μM bleomycin or 175 μM zeocin for 1 h, and then allowed them to be recovered from the DNA damages for 1 d to 5 d. Histochemical staining analysis revealed that GUS accumulation was strongly enhanced up to 2 d after genotoxin treatments, and gradually decreased until 5 d after genotoxin treatments (Fig. 2). Notably, the induced GUS expression was maintained in

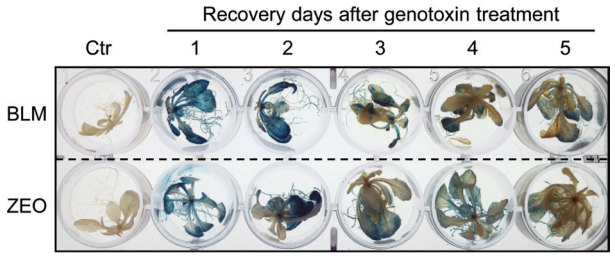

Fig. 2. Response of *AtAGO2* promoter at various recovery days after the treatments with DNA DSBs-inducing genotoxins. Two-week-old seedlings of the *1.3kb$_{pro}$-GUS* overexpression mutant were incubated in a half-strength MS liquid media, supplemented with 1% (w/v) sucrose solution containing 33 μM bleomycin (BLM) or 175 μM of zeocin (ZEO) for 1 h. After 1, 2, 3, 4, and 5 d of incubation in the MS medium in the absence of the genotoxins, the seedlings were stained for the visualization of GUS expression. Ctr, no treatment.

the higher levels in comparison to that of γ-irradiation, but the GUS expression level was relatively higher in the zeocin treatment.

3. The response of *AtAGO2* promoter to mitomycin C and methyl methanesulfonate

To examine whether the expression of *AtAGO2* is induced by a different molecular event other than DNA damages (e.g., DNA DSBs), we employed mitomycin C (MMC) and methyl methanesulfonate (MMS), both of which are associated with the inhibition of DNA replication (Menke *et al*. 2001; Chankova *et al*. 2007; Kozak *et al*. 2009). The transgenic *Arabidopsis* mutants expressing *1.3kb$_{pro}$-GUS* were challenged with 0.5 mM mitomycin C or 5 mM methyl methanesulfonate for 2 h, and the staining of GUS protein were subsequently observed using the histochemical analysis. While positive control plants overexpressing *GUS* gene under the control of 35S promoter showed strong GUS expression in control, MMC, or MMS treatments, no GUS staining was observed in the *1.3kb$_{pro}$-GUS* mutants in response to all the treatments (Fig. 3A). Notably, the MMC treatment elicited the expression of GUS, but the expression level was negligible. RT-PCR analyses confirmed that ectopic *GUS* expression was not induced by MMC and MMS treatments, with the weak increase of endogenous *AtAGO2* against MMC treatment (Fig. 3B).

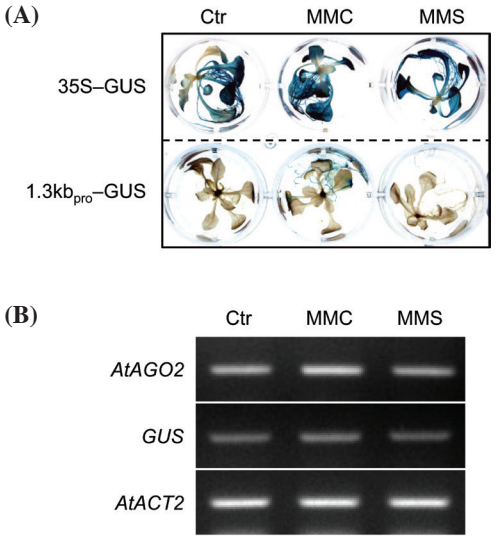

Fig. 3. Gene expression pattern of *AtAGO2* promoter in response to mitomycin C (MMC) and methyl methanesulfonate (MMS). Two-week-old seedlings of the *1.3kb$_{pro}$-GUS* overexpression mutant were incubated in a half-strength MS liquid medium, supplemented with 1% (w/v) sucrose containing 0.5 mM MMC or 5 mM MMS for 2 h. Then the seedlings were subsequently stained for the visualization of GUS expression, or transcript levels were examined. (A) Histochemical GUS staining, (B) Transcript levels of *AtAGO2* and *GUS*. Ctr, no treatments; 35S-GUS, a positive control of GUS expression; *AtACT2*, an endogenous control gene.

DISCUSSION

We investigated the promoter of the *AtAGO2* gene, whose expression is induced by γ-irradiation (Wei *et al*. 2012), to characterize its transcriptional regulation depending on the **r**ecovery days **a**fter the **i**rradiation (RAI). We constructed the upstream sequence (1,313 bp) of *AtAGO2* from the 5′ end fused with *GUS* as a reporter gene (Fig. 1A). The GUS protein of the transgenic *Arabidopsis* plants (*1.3kb$_{pro}$-GUS*) was strongly induced at 1 RAI, but decreased from 3 RAI, and no GUS staining was observed at 5 RAI (Fig. 1B). The transcript levels of endogenous *AtAGO2* and ectopic *GUS* genes were immediately increased after γ-irradiation, however, the transcript levels were decreased from 1 RAI (Fig. 1C). These results indicate that the recovery of DNA damages derived from γ-irradiation was readily recovered after 1 RAI. The GUS staining at 1 RAI would reflect the time for translation from induced GUS transcripts, and the staining at 2 RAI could be explained with a half-life of GUS protein in living mesophyll cells of $<$50 h (Jefferson *et al*. 1987).

We investigated the expression pattern of the *AtAGO2* promoter in response to DSB-inducing genotoxic agents, bleomycin and zeocin (Fig. 2). Genotoxin treatments strongly induced GUS expression in the transgenic plants (*1.3kb$_{pro}$-GUS*), and the effect was more significant in zeocin treatment compared with bleomycin treatment. The expression levels were decreased by the extension of the recovery days, indicating that a process of DNA damage repair system reduced the expression of the *AtAGO2*.

We further investigated the ability of the *AtAGO2* promoter to be activated by the treatments of other genotoxic agents, MMC and MMS, which make replication blocks and thus inhibit DNA replication. No GUS expression was observed with the treatment of mitomycin C and methyl methanesulfonate (Fig. 3). Together, these results suggest that DNA DSBs would be a major effector to elicit the *AtAGO2* promoter activation, however, further characteri-

zations of the promoter will be required using the reagents causing different types of DNA damages.

CONCLUSION

The present study shows that the expression of the *AtAGO2* is significantly induced by γ-irradiation, bleomycin and zeocin, which elicit mainly DNA DSBs. These results are indirectly confirmed by determining the decrease of the *AtAGO2* and *GUS* expression during the recovery days of DNA damages after the treatments of γ-rays and genotoxins. However, the MMC and MMS treatments, which inhibit DNA replication, have no effects on the activation of the *AtAGO2* expression. Therefore, we conclude that DNA DSBs is one of main signals to induce the *AtAGO2* expression.

ACKNOWLEDGMENT

This work was supported by the Nuclear R & D Program of the Ministry of Science, ICT and Future Planning (MSIP), Republic of Korea.

REFERENCES

Bechtold N and Pelletier G (1998) In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. In: Martinez-Zapater JM, Salinas J (eds) Arabidopsis Protoc. Humana Press, Totowa, NJ, pp 259-266. doi:10.1385/0-89603-391-0:259.

Bohmert K, Camus I, Bellini C, Bouchez D, Caboche M and Benning C (1998) *AGO1* defines a novel locus of *Arabidopsis* controlling leaf development. *EMBO J*. **17**(1):170-180.

Chankova SG, Dimova E, Dimitrova M and Bryant PE (2007) Induction of DNA double-strand breaks by zeocin in *Chlamydomonas reinhardtii* and the role of increased DNA double-strand breaks rejoining in the formation of an adaptive response. *Radiat. Environ. Biophys*. **46**(4):409-416.

Garcia-Ruiz H, Carbonell A, Hoyer JS, Fahlgren N, Gilbert KB, Takeda A, Giampetruzzi A, Garcia Ruiz MT, McGinn MG, Lowery N, Martinez Baladejo MT and Carrington JC (2015) Roles and programming of Arabidopsis ARGONAUTE proteins during *Turnip mosaic virus* infection. *PLoS pathogens* **11**(3):e1004755.

Jefferson RA, Kavanagh TA and Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J*. **6**(13):3901-3907.

Karimi M, Inzé D and Depicker A (2002) GATEWAY^{TM} vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci*. **7**(5):193-195.

Kim JH, Cho EJ, Lee MH, Kim JE, Chung BY, Kim TH and Kim J-H (2010) Differential modulation of volatile constituents in *Artemisia princeps* and *Artemisia argyi* plants after gamma ray or electron beam irradiation. *J. Radiat. Ind*. **6**(1): 17-21.

Kozak J, West CE, White C, da Costa-Nunes JA and Angelis KJ (2009) Rapid repair of DNA double strand breaks in *Arabidopsis thaliana* is dependent on proteins involved in chromosome structure maintenance. *DNA Repair* **8**(3):413-419.

Mallory A and Vaucheret H (2010) Form, function, and regulation of ARGONAUTE proteins. *Plant Cell* **22**(12):3879-3889.

Menke M, Chen IP, Angelis KJ and Schubert I (2001) DNA damage and repair in *Arabidopsis thaliana* as measured by the comet assay after treatment with different classes of genotoxins. *Mutat. Res*. **493**(1-2):87-93.

Morel JB, Godon C, Mourrain P, Beclin C, Boutet S, Feuerbach F, Proux F and Vaucheret H (2002) Fertile hypomorphic *ARGONAUTE* (*ago1*) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* **14**(3): 629-639.

Parker JS (2010) How to slice: snapshots of Argonaute in action. *Silence* **1**(1):1-10.

Qu F, Ye X and Morris TJ (2008) *Arabidopsis* DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. *Proc. Natl. Acad. Sci. U.S.A*. **105**(38):14732-14737.

Raja P, Jackel JN, Li S, Heard IM and Bisaro DM (2014) *Arabidopsis* double-stranded RNA binding protein DRB3 participates in methylation-mediated defense against geminiviruses. *J. Virol*. **88**(5):2611-2622.

Song J and Bent AF (2014) Microbial pathogens trigger host DNA double-strand breaks whose abundance is reduced by plant defense responses. *PLoS pathogens* **10**(4):e1004030.

Vaucheret H (2008) Plant ARGONAUTES. *Trends Plant Sci*. **13**(7):350-358.

Wei W, Ba Z, Gao M, Wu Y, Ma Y, Amiard S, White CI, Rendtlew Danielsen JM, Yang Y-G and Qi Y (2012) A role for small RNAs in DNA double-strand break repair. *Cell* **149** (1):101-112.

Zhang X, Yuan Y-R, Pei Y, Lin S-S, Tuschl T, Patel DJ and Chua N-H (2006) *Cucumber mosaic virus*-encoded 2b

suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. *Genes Dev.* **20**(23):3255-3268.

Zhang X, Zhao H, Gao S, Wang W-C, Katiyar-Agarwal S, Huang H-D, Raikhel N and Jin H (2011) *Arabidopsis* Argonaute 2 regulates innate immunity via miRNA393-mediated silencing of a golgi-localized SNARE gene, *MEMB12*. *Mol. Cell* **42**(3):356-366.

Received: 15 October 2016
Revised: 21 November 2016
Revision accepted: 5 December 2016