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ATM-mediated phosphorylation of SOG1 is essential for the DNA damage response in *Arabidopsis*Kaoru O. Yoshiyama^{1,2+}, Junya Kobayashi³, Nobuo Ogita¹, Minako Ueda¹, Seisuke Kimura², Hisaji Maki¹ & Masaaki Umeda^{1,4++}¹Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara, ²Department of Bioresource and Environmental Sciences, Kyoto Sangyo University, Kyoto, ³Radiation Biology Center, Kyoto University, Kyoto, and ⁴JST, CREST, Nara, Japan

***Arabidopsis* SOG1 (suppressor of gamma response 1) is a plant-specific transcription factor that governs the DNA damage response. Here we report that SOG1 is phosphorylated in response to DNA damage, and that this phosphorylation is mediated by the sensor kinase ataxia telangiectasia mutated (ATM). We show that SOG1 phosphorylation is crucial for the response to DNA damage, including transcriptional induction of downstream genes, transient arrest of cell division and programmed cell death. Although the amino-acid sequences of SOG1 and the mammalian tumour suppressor p53 show no similarity, this study demonstrates that ATM-mediated phosphorylation of a transcription factor has a pivotal role in the DNA damage response in both plants and mammals.**

Keywords: DNA damage; *Arabidopsis*; SOG1; phosphorylation; ATM

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INTRODUCTION

Chromosomal DNA constantly suffers many types of damage caused by the action of exogenous agents such as ionizing radiation, ultraviolet and chemical mutagens, or by cellular metabolism, which produces reactive radicals and stalled replication forks. In response to DNA damage, eukaryotic cells activate signalling pathways that stimulate DNA repair, cell cycle checkpoints, and eventually apoptosis; these events are critical for the maintenance of genome integrity [1]. In mammals, DNA damage is sensed by two protein kinases, ataxia telangiectasia

mutated (ATM) and ATM and Rad3-related (ATR), which belong to the phosphatidylinositol 3-kinase family [2]. Each is activated in response to different types of DNA damage: ATM responds to double-strand breaks (DSBs), whereas ATR mainly responds to single-strand DNA and stalled replication forks [3,4]. Checkpoint kinases 1 and 2 (CHK1, CHK2) are key signal transducers working downstream of ATR and ATM, respectively, and p53 transcription factor is another crucial downstream factor controlling DNA repair, cell cycle arrest, senescence and apoptosis [5,6].

Arabidopsis also possesses ATM and ATR orthologs, which are not essential under non-stressed conditions, although *atm* knockout mutants show partial sterility [7,8]. This is in marked contrast to mammalian ATR, whose disruption induces embryonic lethality [9]. In *Arabidopsis*, *atm* and *atr* mutants are hypersensitive to DSB-inducing agents and replication-blocking agents, respectively [7,8]. Such hypersensitivity is similar to that of mammalian mutants of ATM and dominant negative mutants of ATR [3,4], indicating conserved functions among plants and mammals. However, counterparts of the signal transducers CHK1, CHK2 and p53 are absent in *Arabidopsis*, suggesting that plants deploy a unique system to transmit the DNA damage signal downstream.

We have previously reported that *Arabidopsis* SOG1 is a plant-specific transcription factor that governs gene transcription, cell cycle arrest and programmed stem-cell death in response to DNA damage, and maintains genomic stability [10,11]. Although the functions of SOG1 are similar to those of mammalian p53, the two proteins' amino-acid sequences are unrelated to each other (Fig 1A). Therefore, it would be intriguing to establish how SOG1 function is controlled in response to DNA damage. Here we demonstrate that serine-glutamine motifs in SOG1 are phosphorylated in an ATM-dependent and ATR-independent manner in response to DSBs. This phosphoregulation is a prerequisite to signal transduction triggered by DSBs, implying an essential role for the ATM–SOG1 pathway in plants.

RESULTS AND DISCUSSION
SOG1 localization in plants

The *sog1-1* mutant was isolated as a suppressor of the γ -sensitive phenotype displayed by seeds defective for the repair endonuclease

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Fig 1 | Expression pattern of SOG1. (A) Functional domains of p53 and SOG1. Ser15 and Ser20 in p53 are phosphorylated in response to DNA damage. Serine residues of the five SQ motifs in the C-terminal region of SOG1 are shown. (B–F) GUS staining of 4-day-old seedlings expressing *pSOG1::SOG1-GUS*. Whole seedlings (B), cotyledon (C), root tip (D), lateral root primordium (E) and root stele (F). (G) Microscopic observation of GFP fluorescence in a 4-day-old root tip expressing *pSOG1::SOG1-GFP*. The root was counterstained with propidium iodide to visualize the cell wall. The inset shows a magnified image of the indicated region. Scale bars, 1 mm (B,C) and 0.1 mm (D–G). GUS, β -glucuronidase; SOG1, suppressor of gamma response 1; SQ, serine-glutamine.

XPF; thus, the transcriptional response to DNA damage (assessed by monitoring the expression of two DNA repair-related genes, *BRCA1* and *RAD51A*) is impaired in *xpf-2 sog1-1* (supplementary Fig S1 online) [11,12]. To determine the localization of SOG1 protein in plants, we generated transgenic *Arabidopsis* plants carrying *pSOG1::SOG1-GUS* (*SOG1- β -glucuronidase*), in which the promoter and coding regions of *SOG1* are fused in-frame to the GUS reporter gene. The transgene could partially restore the abolished transcriptional response to DNA damage in *xpf-2 sog1-1*: expression of both *BRCA1* and *RAD51A* was stimulated by a DSB inducer, zeocin, in transgenic plants (supplementary

Fig S1 online). We observed strong GUS staining in meristematic tissues, such as the shoot and root apical meristems, and in lateral root primordia in 4-day-old seedlings (Fig 1B–E). Faint GUS staining was also observed in the vasculature of young leaves and in the root stele (Fig 1B,C,F). Because the tissues in which GUS staining was observed all have dividing cells [13], we conclude that SOG1 functions mainly in tissues displaying cell division activity.

We assessed the subcellular localization of SOG1 using transgenic plants carrying *pSOG1::SOG1-GFP* (*SOG1-green fluorescent protein*), which complemented the defective transcriptional response of *sog1-1* to zeocin (supplementary Fig S1 online). As shown in Fig 1G, SOG1-GFP protein was localized exclusively to the nucleus. In addition to the observation that *SOG1* mRNA (messenger RNA) level does not increase after ionizing radiation treatment [14], our analysis showed that neither intensity nor localization of the SOG1-GFP signal were affected by 100 μ M zeocin treatment in 5-day-old seedlings (supplementary Fig S2 online). This indicates that SOG1 function is not controlled by protein accumulation or subcellular localization.

SOG1 is phosphorylated in response to DNA damage

In mammals, the preferred target for phosphorylation by ATM and ATR is serine or threonine followed by glutamine [4]. As SOG1 has five serine-glutamine (SQ) motifs in its C-terminal region (Fig 2A), we predicted that SOG1 would likewise be modified by ATM and/or ATR, and thereby activated, in response to DNA damage. To test this possibility, we first prepared polyclonal antibodies against SOG1, but their specificity was too low to detect endogenous SOG1. We therefore generated *Arabidopsis* transgenic plants expressing 10xMyc-tagged *SOG1* under its own promoter. The SOG1-Myc fusion protein was functional, as it complemented the defective transcriptional response of *sog1-1* to zeocin (supplementary Fig S1 online).

Immunoblotting with anti-Myc antibody detected an 80-kDa protein in transgenic plants expressing *SOG1-Myc* but not in wild-type (WT) plants (Fig 2B), indicating that the antibody properly recognizes the fusion protein. The discrepancy between this molecular mass and the estimated value of 66 kDa might be due to the low pI (4.62) of SOG1-10xMyc. To investigate whether SOG1 is modified in response to DNA damage, seedlings expressing *SOG1-Myc* were unirradiated (0 Gy) or γ -irradiated at 50 Gy, and, 1 hour later, total protein was extracted from roots. Immunoblots of protein extract from unirradiated plants showed a slower migrating band (band 'b') in addition to the 80-kDa protein (band 'a'), whereas in irradiated plants a third, more slowly migrating band (band 'c') appeared (Fig 2C). Similar results were also obtained by zeocin treatment, in a dose-dependent manner (supplementary Fig S3 online). Bands b and c both disappeared when protein extracts were incubated with λ protein phosphatase, but not with λ protein phosphatase and its inhibitor (Fig 2D). These results indicate that a small amount of SOG1 is phosphorylated even without genotoxic stress, and that part of the SOG1 population becomes hyperphosphorylated in response to DSBs. In mammals, p53 level is kept low under unstressed conditions by protein degradation, whereas it is raised under genotoxic stress conditions [15]. However, we could not see any substantial change of the SOG1-Myc level in immunoblotting on γ -irradiation or zeocin treatment (Fig 2C; supplementary Fig S3 online), which is consistent with the result obtained for the SOG1-GFP fusion

Fig 2 | *In vivo* phosphorylation of SOG1. (A) Amino-acid sequence of SOG1. Blue and red letters show the NAC (NAM, ATAF1, 2 and CUC2) domain and the SQ motifs, respectively. (B) Immunoblotting of total protein with anti-Myc antibody (upper panel). Total protein was extracted from WT and transgenic plants expressing *pSOG1::SOG1-Myc* (WT/*SOG1-Myc*). As a control, the same membrane was incubated with anti-CDKA;1 antibody (lower panel). (C,D) Plants harbouring *pSOG1::SOG1-Myc* were untreated (0 Gy) or treated with γ -irradiation (50 Gy), and total protein was extracted an hour later. Total protein was incubated with λ PP in the presence or absence of phosphatase inhibitor (D). ‘-’ indicates controls without λ PP. Phosphorylated forms of SOG1 were detected in an SDS-PAGE gel containing Phos-tag. The exposure time was 10 or 40 s in (D). (E,F) Plants harbouring *pSOG1::SOG1-Myc* or *pSOG1::SOG1(5A)-Myc* were used. Five-day-old seedlings grown on MS plates were transferred to liquid medium with (+) or without (-) 1 mM zeocin, and total protein was extracted an hour later. The protein extracts were separated in an SDS-PAGE gel containing Phos-tag. To ensure that transferring seedlings from solid to liquid had no effect on SOG1 phosphorylation, total protein was also extracted from seedlings on MS plates as a n.t. In the right panel of (E), five times more total protein was used for *atm-2* and *atr-2* than for WT to obtain comparable loading of SOG1-Myc. Coomassie blue staining is displayed below. Non-phosphorylated, phosphorylated and hyperphosphorylated SOG1-Myc (bands a, b and c, respectively) are indicated by arrowheads. MS, Murashige and Skoog; n.t., non-transferred, PP, protein phosphatase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SOG1, suppressor of gamma response 1; SQ, serine-glutamine; WT, wild type.

protein (supplementary Fig S2 online). Therefore, unlike mammalian p53, SOG1 function is probably mainly controlled by phosphorylation, rather than by protein accumulation. We could detect the hyperphosphorylated SOG1 after 1 hour of γ -irradiation or zeocin treatment (Fig 2C; supplementary Fig S3 online), suggesting that rapid modification of this transcription factor is involved in the DNA damage response.

Hyperphosphorylation of SOG1 is dependent on ATM

Both SOG1 and ATM are required for the transcriptional response following γ -irradiation, suggesting that these two proteins act in the same pathway [11,14]. We therefore examined whether SOG1 phosphorylation after zeocin treatment is altered in the *atm-2* mutant. In *atm-2* carrying *pSOG1::SOG1-Myc*, the transcript level of endogenous *SOG1* was similar to that in WT, but the level of *SOG1-Myc* transgene messenger RNA (mRNA) was markedly lower than that in WT plants expressing *SOG1-Myc* (supplementary

Fig S4 online). This might be because the *atm-2* line is heterozygous for the *SOG1-Myc* transgene. As a result, the protein level of SOG1-Myc was reduced in *atm-2*; thus, to detect the phosphorylated forms, we exposed the immunoblots for a longer period (Fig 2E). We found that hyperphosphorylated SOG1 (band ‘c’) was missing in zeocin-treated *atm-2*, but that DSB-independent phosphorylation represented by band b was observed in both WT and *atm-2* (Fig 2E, left panel). This result demonstrates that DNA damage-induced hyperphosphorylation of SOG1 is ATM dependent, whereas the constitutive phosphorylation is attributable to another kinase, which is not associated with the DNA damage response. We also tested the *atr-2* mutant, and found that hyperphosphorylated SOG1 (band ‘c’) appeared in zeocin-treated seedlings (Fig 2E, right panel), indicating that ATR does not have a main role in zeocin-induced SOG1 phosphorylation.

To examine whether SOG1 is also hyperphosphorylated in response to replication stress, we used the two replication-blocking

agents, hydroxyurea (a ribonucleotide reductase inhibitor) and aphidicolin (an inhibitor of DNA polymerase α). SOG1 was not hyperphosphorylated after up to 24 h of hydroxyurea treatment (supplementary Fig S5 online). Aphidicolin treatment yielded a trace amount of hyperphosphorylated SOG1 (supplementary Fig S5 online), which might be a consequence of DSBs that were indirectly induced by replication block. These results indicate that SOG1 is hyperphosphorylated in response to DSBs, but not to replication stress.

To elucidate the putative phosphorylation sites, we transformed the *sog1-1* mutant with *SOG1-Myc* or *SOG1(5A)-Myc*, the latter of which encodes serine-to-alanine substitutions at all five SQ motifs. Hyperphosphorylated SOG1 (band 'c') was observed in zeocin-treated plants carrying *SOG1-Myc* but not *SOG1(5A)-Myc* (Fig 2F), indicating that one or more of the SQ motifs in the C-terminal region are targets for ATM-dependent phosphorylation. In contrast, band b was detected for both *SOG1-Myc* and *SOG1(5A)-Myc* regardless of zeocin treatment (Fig 2F), showing that DSB-independent phosphorylation of SOG1 does not involve the SQ motifs.

hATM phosphorylates the SQ motifs of SOG1 *in vitro*

Human ATM (hATM) phosphorylates p53 *in vitro*, and this kinase activity is markedly enhanced after treatment of cells with a radiomimetic drug [16]. Because the phosphatidylinositol 3-kinase domain of *Arabidopsis* ATM shares 67% amino-acid similarity with that of hATM [17], we investigated whether ATM directly phosphorylates SOG1. As we did not have a satisfactory antibody against *Arabidopsis* ATM, we immunoprecipitated endogenous hATM from human lymphoblastoid cells using a commercial anti-ATM antibody. For the kinase assay, the hATM immunoprecipitates were incubated with recombinant glutathione S-transferase (GST)-SOG1 or GST-p53 as substrate. As expected, GST-p53 was phosphorylated by hATM, and higher phosphorylation was observed for hATM from cells that had been activated by γ -irradiation (supplementary Fig S6A, lanes 1 and 2 online). Similarly, GST-SOG1 was phosphorylated by the hATM immunoprecipitates, and the phosphorylation level was also elevated by γ -irradiation, although the enhancement was not as marked as for GST-p53 (supplementary Fig S6A, lanes 3 and 4 online). No phosphorylation was observed when GST-SOG1 was incubated with immunoprecipitates prepared using immunoglobulin G, or when GST-SOG1(5A) carrying the serine-to-alanine substitutions was incubated with the hATM immunoprecipitates (supplementary Fig S6A, lanes 5 and 6 online). However, the phosphorylation was observed when GST-SOG1(3A) (with 350Ala, 356Ala and 372Ala) or GST-SOG1(2A) (with 430Ala and 436Ala) was used as substrate (supplementary Fig S6C online), indicating that multiple SQ sites are direct targets of hATM. The phosphorylation level of GST-SOG1(3A) or (2A) was much higher than that of GST-SOG1(WT). As GST forms a dimer under physiological conditions [18], GST-SOG1(WT) might not be effectively phosphorylated owing to altered conformation; conversely, alanine substitutions might render GST-SOG1(3A) or (2A) more easily accessible to hATM.

The SQ phosphorylation is required for SOG1 function

To reveal the physiological significance of DNA damage-induced phosphorylation of SOG1, we tested the ability of *SOG1(5A)-Myc* to complement the phenotype of *sog1-1*. *SOG1-Myc* suppressed

Table 1 | Phosphorylation of the SQ motif is required for γ -sensitive growth in *xpf-2*

Line	Genotype	Total	No. γ -resistant (<i>xpf-2</i> <i>sog1-1</i> phenotype)	No. γ -sensitive (<i>xpf-2</i> <i>SOG1</i> phenotype)	% γ -sensitive
KS12	Wild type	35	35	0	0
KO711	<i>xpf-2</i>	23	0	23	100
KO735	<i>xpf-2 sog1-1</i>	44	44	0	0
KS90	<i>xpf-2 sog1-1</i> / <i>SOG1-Myc</i>	32	3	29	91
KS70	<i>xpf-2 sog1-1</i> / <i>SOG(5A)-Myc</i>	31	31	0	0

MS, Murashige and Skoog; SOG1, suppressor of gamma response 1; SQ, serine-glutamine. Imbibed seeds were γ -irradiated (100 Gy), and grown on MS plates for 10 days. The number of plants that were γ -resistant (plants having one or more true leaves) and γ -sensitive (plants with no true leaves) was scored by eye. If a transgene is functional, the γ -sensitive (*xpf-2 SOG1*) phenotype appears. Note that all unirradiated seedlings formed true leaves.

the γ -resistant leaf phenotype of the *xpf-2 sog1-1* mutant, which is defective in programmed developmental arrest on DNA damage [12]; in contrast, *SOG1(5A)-Myc* could not complement this phenotype at all (Table 1). Similarly, transcriptional induction of *BRCA1* and *RAD51A* on zeocin treatment was restored in *sog1-1* carrying *SOG1-Myc* but not *SOG1(5A)-Myc* (Fig 3A). It is known that DSBs lead to SOG1-dependent cell death in the stem cell region of roots (Fig 3B, arrowhead) [10]. Our observation of zeocin-treated roots showed that *SOG1-Myc* but not *SOG1(5A)-Myc* rescued the impaired cell death phenotype in *sog1-1* (Fig 3B). Taken together, these results lead us to conclude that ATM-dependent phosphorylation of the SQ motifs is essential for SOG1 to exert its function in cell cycle arrest, the transcriptional response and cell death induction in response to DSBs. The SQ motifs reside in the transcription activation domain of SOG1 (Fig 1A) [19]; thus, it is possible that phosphorylation of this domain affects the transcription-inducing activity of SOG1 or its interaction with cofactors that control the affinity and/or selectivity of SOG1 for target genes.

Although sensor proteins (ATM and ATR) for DNA damage are conserved between mammals and plants, the transcription factors (p53 and SOG1) that transmit the signals to downstream regulators have diverged. However, here we report the remarkable functional similarity that both p53 and SOG1 are phosphoregulated by ATM [16]. p53 is also known to be phosphorylated and activated by CHK2 [20]; although plants have no CHK2 homologue, our results show that SOG1 is constitutively phosphorylated by an unknown kinase, and that this phosphorylation is independent of DNA damage, ATM or the SQ motif. Further studies should show how kinase(s) other than ATM control SOG1 function in contexts such as development and the stress response.

The growth strategies of animals and plants are quite different; for example, plant cells have rigid cell walls, and therefore cannot migrate. Such differences have probably resulted in specific adjustments to their DNA damage response. We previously demonstrated that, in the epidermal cells of *Arabidopsis* root tips,

Fig 3 | SQ motif phosphorylation is required for SOG1 function. (A) Semiquantitative RT-PCR of *BRCA1* and *RAD51A*. Five-day-old WT, *sog1-1*, and *sog1-1* carrying *pSOG1::SOG1-Myc* or *pSOG1::SOG1(5A)-Myc* were transferred to fresh MS liquid medium containing 0 (–) or 100 μ M (+) zeocin, and RNA was extracted from root tips 2 h later. As a control, cDNA for *eIF4A1* was amplified. (B) Stem-cell death in zeocin-treated root tips. Four-day-old seedlings were transferred to a new MS plate containing 0 or 100 μ M zeocin, and roots were stained with propidium iodide 24 h later. Arrowheads indicate cell death in the stem cell region. Bar, 100 μ m. cDNA, complementary DNA; MS, Murashige and Skoog; RT-PCR, reverse transcription polymerase chain reaction; SOG1, suppressor of gamma response 1; SQ, serine-glutamine.

DSBs induce an early onset of DNA polyploidization that enables meristematic cells to stop cell division and promotes cell expansion [21]. The inability of cells to migrate might require damaged cells to avoid the strategy of apoptosis, available to animal cells, because, in plants, dead cells are usually not replaced by the surrounding living cells. On the other hand, DSBs induce cell death in the stem cell region of *Arabidopsis* roots where the space left by dead cells can be occupied by new cells generated as the surrounding cells divide [22]. Because SOG1 is required for both DNA polyploidization and cell death in roots [11,20], it is likely to regulate different sets of target genes in a cell type-specific manner. It will be interesting to show how SOG1 phosphorylation is involved in the differential control of target genes and triggers various outputs, such as DNA repair, DNA polyploidization and cell death, to overcome genotoxic stress.

METHODS

Plant materials and growth conditions. *Arabidopsis thaliana* ecotype Columbia (Col) was used as the WT strain. *xpf-2*, *sog1-1*, *xpf-2 sog1-1*, *atm-2* (SALK_006953) and *atr-2* (SALK_032841) have been described previously [7,8,11]. Plants were grown on soil or MS media (1 $\times$ Murashige and Skoog (MS) salts including vitamins, 2% (w/v) sucrose, pH 6.0; 0.8% (w/v) gellan gum agar for solid medium) under continuous light conditions at 23 $^{\circ}$ C. Methods for the generation of transgenic plants and complementation tests are available in the supplementary information online.

Reverse transcriptase polymerase chain reaction. Total RNA was extracted from root tips of 5-day-old seedlings using an RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized from 0.8 μ g of total RNA using Transcriptor Universal cDNA Master (Roche), and used as template for PCR amplification with specific primers. Detailed methods are available in the supplementary information online.

Immunoblotting. Proteins were separated in 8% SDS-polyacrylamide gels and blotted onto polyvinylidene fluoride membranes. SOG1-Myc was detected using anti-Myc antibody

(Santa Cruz). Phosphorylated SOG1-Myc was detected by Phos-tag reagent (NARD Institute). Detailed methods are available in the supplementary information online.

GUS staining and kinase assay. See the supplementary information online for details.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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Author contributions: K.O.Y. planned the experiments and performed the majority of the experiments. J.K. performed the kinase assay. N.O. performed site-directed mutagenesis to generate alanine substitutions in GST-SOG1. M.Ue. generated transgenic plants. K.O.Y., S.K., H.M. and M.Um. conceived the project and wrote the paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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