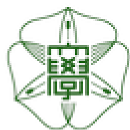

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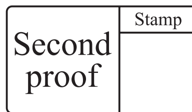

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DRD1, a SWI/SNF-like chromatin remodeling protein, regulates a heat-activated transposon in *Arabidopsis thaliana*

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ONSEN is a heat-activated LTR retrotransposon in *Arabidopsis thaliana*. Screens to identify transcriptional regulatory factors of *ONSEN* revealed a SWI/SNF-like chromatin remodeling protein, DRD1, which cooperates with plant-specific RNA polymerase and is involved in RNA-directed DNA methylation. *ONSEN* transcript level was increased in the *drd1* mutant relative to wild-type under heat stress, indicating that DRD1 plays a significant role in the silencing of activated *ONSEN* under the stress condition. The transcript level of *HsfA2*, which is directly involved in transcriptional activation of *ONSEN*, was not higher in the *drd1* mutant than in the wild-type. Interestingly, no transgenerational transposition of *ONSEN* was observed in the *drd1* mutant, even though DNA methylation levels were significantly reduced and expression levels were increased compared to the wild-type. These results suggest that other factors are involved in the regulation of *ONSEN* transposition in addition to the transcript level of *ONSEN*.

Key words: heat stress, DRD1, transposon, *ONSEN*, RNA-directed DNA methylation

INTRODUCTION

Transposable elements (TEs) of various classes, including both DNA transposons and retrotransposons, are abundant in plant genomes and impact genome evolution and gene expression (Wessler, 1996; Kumar and Bennetzen, 1999; Kazazian, 2004; Makarevitch et al., 2015). Despite their potential to either disadvantage or benefit the host genome, most TEs are silent and rarely transpose due to genetic aberrations such as point mutations, deletions or recombination that disrupt their activities. Even though full-length autonomous transposons are intact and have the ability to transpose, host plants have evolved various types of epigenetic regulation, such as DNA methylation or histone modification, to defend their genome against such transposition.

One of the well-studied mechanisms for TE regulation is RNA-directed DNA methylation (RdDM), in which small interfering RNAs (siRNAs) direct the cytosine methylation of DNA sequences that are complementary to the siRNAs (Wierzbicki et al., 2008; Gao et al., 2010). RdDM requires transcriptional machinery that involves two plant-specific RNA polymerases, RNA polymerase IV (Pol IV) and RNA polymerase V (Pol V) (Kanno et al., 2005b; Onodera et al., 2005; Pontier et al., 2005). Pol IV generates primary RNA transcripts and Pol V generates non-coding transcripts to introduce siRNA-mediated DNA methylation on the target site. In *Arabidopsis thaliana*, RNA-dependent RNA polymerase 2 (RDR2) converts a transcript produced by Pol IV to double-stranded RNA, and DICER-LIKE 3 (DCL3) subsequently processes these precursor RNAs into 24-nt siRNAs (Zhang et al., 2007; Mosher et al., 2008). The siRNAs bind to an RNA-induced silencing complex, RISC, that contains ARGONAUTE 4 (AGO4). AGO4 interacts with Pol V and recruits the DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), which

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directs *de novo* DNA methylation of target TEs (Cao and Jacobsen, 2002; Matzke and Birchler, 2005).

Although most TEs have their own modes of regulation, some are activated under stress conditions (Chandler and Walbot, 1986; Bennetzen, 1987; Hirochika, 1993; Grandbastien et al., 1997, 2004; Scortecci et al., 1997; Steward et al., 2000; Hashida et al., 2003; Henderson and Jacobsen, 2007; Hirayama et al., 2009; Lisch, 2009; Zeller et al., 2009). The stress might induce a change in the epigenetic state of the TE, generating stress-responsive elements in host genes in the proximity of TEs. Epigenomic diversity may allow phenotypic plasticity and the ability to cope with environmental variation.

Previously, we found heat stress-induced activation of a Ty1/copia-like retrotransposon named *ONSEN* in *A. thaliana* (Ito et al., 2011). The activation of *ONSEN* requires a heat stress transcription factor, HsfA2, which distinctively binds to a *cis*-regulatory sequence (heat response element, HRE) in the promoter of the *ONSEN* LTR (Cavrak et al., 2014). Furthermore, the activated *ONSEN* is transposed in stressed plants that are defective in the RdDM pathway (Ito et al., 2011; Matsunaga et al., 2012).

In this study we performed a genetic screening to investigate the regulation mechanism of *ONSEN* and revealed that an epigenetic regulator, DRD1 (defective in RNA-directed DNA methylation), regulates *ONSEN* expression. DRD1 is a member of the plant-specific subfamily of SWI2/SNF2-like proteins (Kanno et al., 2004, 2005b). It associates with many subunits of the Pol V complex and is required for the accumulation of Pol V-dependent transcripts to facilitate RdDM and gene silencing of homologous DNA sequences (Kanno et al., 2005a; Law et al., 2010). Although the activation of *ONSEN* has been studied in some mutants (Ito et al., 2011; Matsunaga et al., 2012), the precise mechanism of transcriptional regulation remains unknown. Here, we provide new insights into chromatin remodeling protein-mediated regulation of *ONSEN*.

MATERIALS AND METHODS

Plant material and stress treatments The *A. thaliana* plants used in the experiments included wild-type Columbia-0 (Col-0) and Landsberg *erecta* (L*er*), mutants *nrpd1-3* (Herr et al., 2005) and *drd1-6* (Kanno et al., 2004), and transgenic plants that possessed a full-length LTR (genome position: 4212570–4213146) of *ONSEN* (*AT5G13205*) fused with a GFP gene (Matsunaga et al., 2015). The plants were grown on Murashige and Skoog medium under continuous light at 21 °C. For heat stress treatment to analyze *ONSEN* expression and DNA methylation, seven-day-old seedlings were subjected to a temperature shift from 21 °C to 37 °C for 24 h. The transcript level of *HsfA2* was analyzed on seven-day-old seedlings that were subjected to a temperature shift from 21 °C to 37 °C for 1 h.

Real-time PCR Total RNA was extracted from seedlings using TRI Reagent (Sigma-Aldrich), according to the supplier's recommendations. Five individual plants were pooled prior to RNA extraction. Around 3 to 5 μg of total RNA was treated with RQ1 RNase-free DNase (Promega) and reverse-transcribed using the ReverTraAce qPCR RT Kit (Toyobo) with random primers. Real-time PCR was performed using the Applied Biosystems 7300 Real-Time PCR System with the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). Three biological repetitions were performed and standard deviation was determined.

Southern blot analysis Plant genomic DNA was isolated using the Nucleon PhytoPure DNA Extraction Kit (GE Healthcare). Southern blots were performed as described previously (Miura et al., 2004). We detected hybridization signals in a highly concentrated sodium dodecyl sulfate hybridization buffer (Church and Gilbert, 1984) using a radiolabeled *ONSEN*-specific probe (Supplementary Table S1) that was generated with the Amersham Megaprime DNA Labeling System (GE Healthcare).

Mapping of mutation by whole-genome sequencing To map the gene responsible for the *boil* mutant, the *boil* mutant (Col-0) was first crossed with wild-type (L*er*), and then self-pollinated to produce a segregating population of *boil* lines. Seedlings of this population were heat-stressed at 37 °C for 24 h, and then grown for about two weeks in pots, one group with high expression of GFP signal (High population) as in the *boil* mutant, and the other with weak expression of GFP fluorescence (Low population) as in the GFP:wild-type (Col-0). The true leaves were then sampled together in three pieces. The number of individuals used in the analysis was 22 for the High sample and 23 for the Low sample in *boil1*, and 20 for the High sample and 21 for the Low sample in *boil5*. Genomic DNA was extracted using the Nucleon PhytoPure DNA Extraction Kit (GE Healthcare). The extracted DNA was fragmented by sonication (S220, Covaris) and then used to construct a library using TruSeq DNA Library Prep Kits (Illumina) according to the manufacturer's protocol. The library was sequenced by NextSeq500 (Illumina). The output bcl files were converted to fastq files by bcl2fastq (Illumina). Based on the read sequences, a search for the gene responsible was performed in Mitsucal (Suzuki et al., 2018). For each mutant, the areas where Col-0-type bias was observed were selected. The search conditions were as follows: [number of substitutions: 1–200; substitution rate: 80–100; QV substitution rate: 80–100; number of control substitutions: blank; only mutation positions affecting the

amino acid sequence: checked; type of base substitution: blank].

DNA methylation analysis For bisulfite sequencing analysis, genomic DNA was extracted from seedlings using the Nucleon PhytoPure DNA Extraction Kit. Bisulfite treatment was performed using the MethylCode Bisulfite Conversion Kit (Thermo Fisher Scientific). Bisulfite-treated DNA was amplified by PCR with the EpiTaq HS (Takara Bio). Primers for the analysis are listed in Supplementary Table S1. PCR products were cloned into the pANT vector using the TA-Enhancer Cloning Kit (Nippon Gene), and ten clones were sequenced for the 5′ LTR region in each line. Methylated sites were analyzed using CyMATE (http://www.cymate.org).

RESULTS

Upregulation of *ONSEN* in EMS mutants subjected to heat stress To understand the molecular mechanism of *ONSEN* regulation, we tried to find a new regulatory factor by mutant screening. We used a transgenic Arabidopsis that possessed an intact LTR of *ONSEN* fused with a gene for green fluorescent protein (GFP). The transgenic plants with a single-copy insert were mutagenized by ethyl methanesulfonate and the resulting M2 progeny were screened for mutations. The GFP signals of 5,000 heat-stressed mutants were compared with that of the parental line subjected to heat stress. Twenty-four individual seedlings with stronger GFP signals were self-pollinated and endogenous *ONSEN* expression in their progeny was analyzed by quantitative reverse transcription PCR. More than twice the expression level of *ONSEN* relative to wild-type was observed in 16 of the lines. We named the mutant lines *boils* (burst of *ONSEN* induction lines). We focused on one of the *boils*, *boil5*, which has strong GFP signals in the seedlings (Fig. 1A). We analyzed the expression level of endogenous *ONSEN* in the self-pollinated progeny of *boil5*. The result showed that the expression level of *ONSEN* in

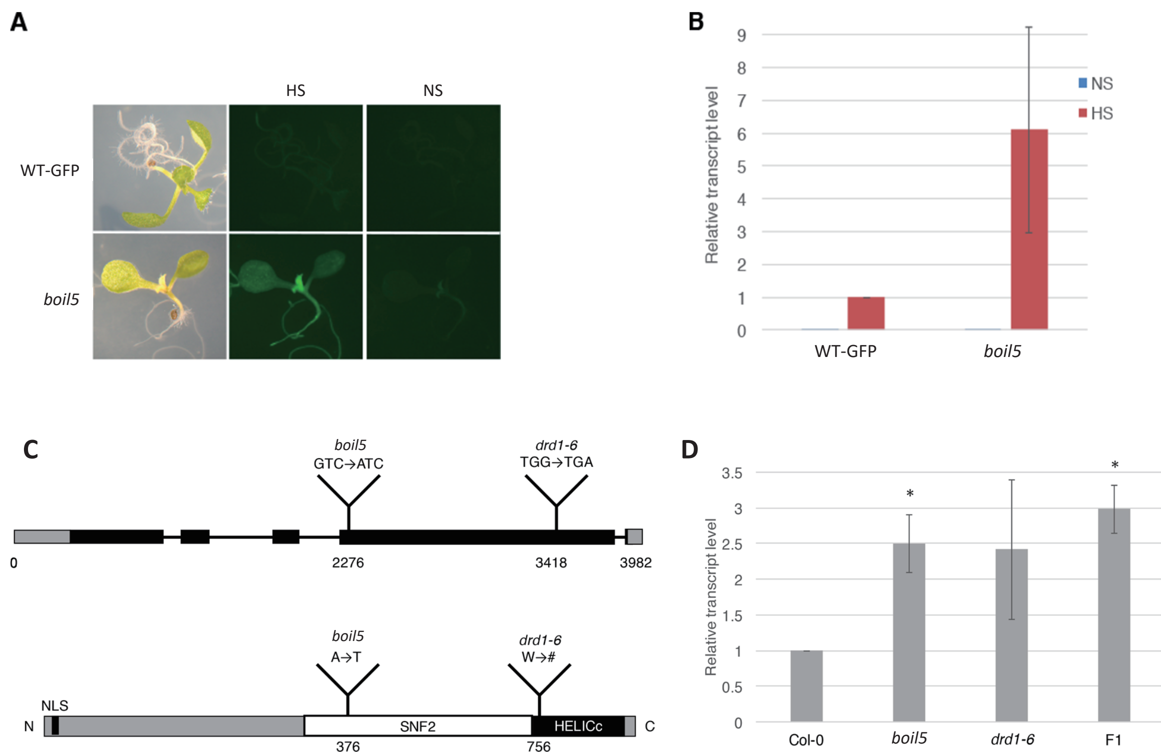

Fig. 1. Activation of *ONSEN* in *boil5* subjected to heat stress. (A) Photographs of bright field and GFP fluorescence images. Wild-type transgenic plants (WT-GFP) and *boil5* plants were observed immediately after heat treatment (HS) or under non-stress (NS) conditions as a control. (B) Relative transcription levels of endogenous *ONSEN*. HS, heat-stressed samples; NS, non-stress samples. Error bars represent the mean ± SEM, $n = 3$; values are relative to heat-stressed transgenic plants (WT-GFP). (C) Structure of the *DRD1* gene and the predicted protein domains. Boxes represent exons, and black and gray colors indicate coding and non-coding regions, respectively, on the gene structure (upper). The positions of the point mutation in *boil5* and *drd1-6* are indicated, together with nucleotide numbers. The amino acid substitutions caused by *boil5* and *drd1-6* are shown in the protein structure (lower) with the predicted amino acid domains, together with amino acid numbers. #, the mutation generates a stop codon. (D) Relative transcript levels of endogenous *ONSEN* in heat-stressed seedlings including wild-type transgenic plants (Col-0), *boil5*, *drd1-6*, and F1 progeny of *boil5* × *drd1-6* (F1). Error bars represent the mean ± SEM, $n = 3$; values are relative to heat-stressed transgenic plants (Col-0). Asterisks mark significant differences from Col-0 ($P \leq 0.05$, Student's *t*-test).

heat-stressed *boil5* was six times higher than that in the parental line (Fig. 1B).

Next, we analyzed the transgenerational transposition of *ONSEN* in *boil*s subjected to heat stress. To detect new inserted copies of *ONSEN* in the progeny of heat-stressed *boil5*, Southern blot analysis was conducted on the next generation in *boil5* plants subjected to heat stress. As a control, *ONSEN* transposition in *boil1* progeny was also analyzed. *boil1* has a mutation in the *NRPD1* gene, which encodes the largest subunit of Pol IV. The mutation causes a non-synonymous substitution in the NRPD1 protein and strong GFP signals in the seedlings (Fig. 2A and 2C). The expression level of *ONSEN* in heat-stressed *boil1* plants was six times higher than that in the parental line (Fig. 2B). Southern blot analysis detected transgenerational transposition of *ONSEN* in the next generation in *boil1* but not in *boil5* subjected to heat stress (Fig. 2D). This result indicated that transcriptional activation was necessary but not sufficient for *ONSEN* transposition.

Mapping of *BOIL5* To identify the gene responsible for *ONSEN* regulation in *boil5,* the mutant was outcrossed with L*er*. Twenty of 134 F2 seedlings showed a strong GFP signal, indicating that the mutation of the gene responsible is recessive. Approximately 20 F2 progeny each that showed high or low expression of GFP were collected, and DNA was extracted from each group in bulk. A DNA library was constructed for each, and sequenced using a high-throughput sequencer. To identify the mutation responsible for the phenotypes of *boil5*, we used the Mitsucal software (Suzuki et al., 2018), which aligned all reads for *boil5* with reference genes by Bowtie with a parameter permitting multiple alignments. Within the 5–10-Mbp region of chromosome 2, 24 mutations with a > 90% ratio of mismatch were detected. Fourteen muta-

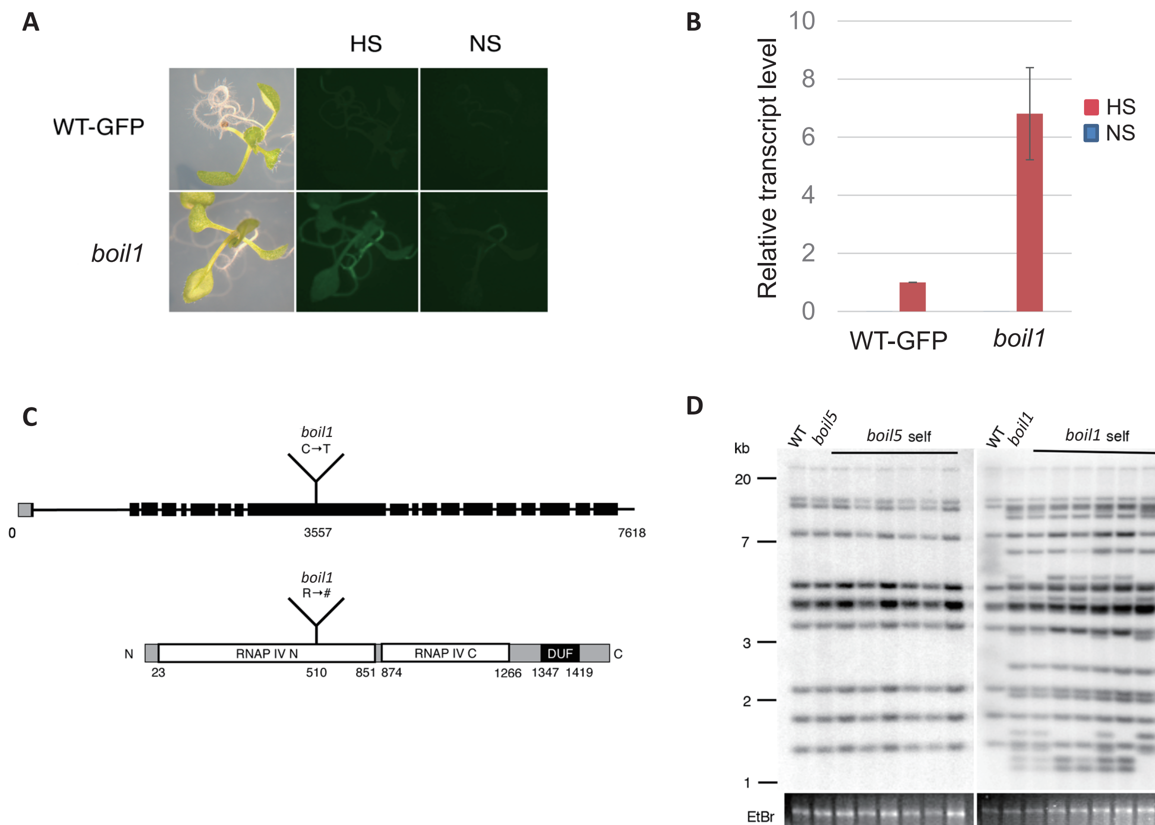

Fig. 2. Activation of *ONSEN* in *boil1* subjected to heat stress. (A) Photographs of bright field and GFP fluorescence images. Wild-type transgenic plants (WT-GFP) and *boil1* plants were observed immediately after heat treatment (HS) or under non-stress (NS) conditions as a control. (B) Relative transcript levels of endogenous *ONSEN*. HS, heat-stressed samples; NS, non-stress samples. Error bars represent the mean ± SEM, n = 3; values are relative to heat-stressed transgenic plants (WT-GFP). (C) Structure of the *NRPD1* gene and the predicted protein domains. Boxes represent exons, and black and gray colors indicate coding and non-coding regions, respectively, on the gene structure (upper). The position of the point mutation in *boil1* is indicated, together with nucleotide numbers. The amino acid substitution caused by *boil1* is shown in the protein structure (lower) with the predicted amino acid domains, together with amino acid numbers. #, the mutation generates a stop codon. DUF, domain of unknown function. (D) Southern blot analysis of *ONSEN* in a heat-stressed *boil1* and *boil5* plant and in self-pollinated siblings. A gel stained with ethidium bromide (EtBr) is shown at the bottom of each panel as a loading control.

tions had no effect on protein structure (mutation in intron, or synonymous substitution). Seven mutations of the remaining ten were a substitution from G to A (or C to T), the most typical mutation caused by EMS. Four mutations were observed only in the F2 progeny that showed high expression of GFP. One of the four was

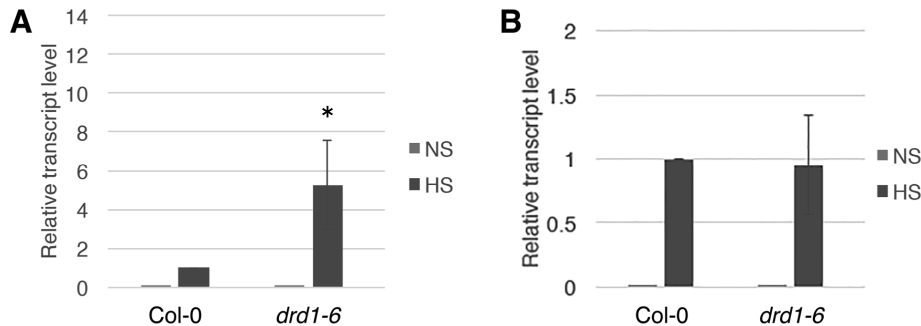

Fig. 3. Relative transcript levels of endogenous *ONSEN* (A) and *HsfA2* (B) in heat-stressed seedlings of wild-type (Col-0) and *drd1-6*. Error bars represent the mean ± SEM, $n = 3$; values are relative to heat-stressed transgenic plants (Col-0). The asterisk marks a significant difference from heat-stressed Col-0 ($P \leq 0.05$, Student's *t*-test).

Fig. 4. Comparison of DNA methylation in wild-type (Col-0) and *drd1-6* in the *ONSEN* 5′ LTR region. (A) DNA methylation was determined by bisulfite-sequencing of the *LTR* region of *At1g11265*. Black and white arrowheads represent individual cytosine methylation status within the analyzed region (black: methylated; white: unmethylated). Circles, squares and triangles indicate CG, CHG and CHH, respectively. (B) DNA methylation level of the LTR region in wild-type (Col-0), *nrpd1-3* and *drd1-6*. Percentage of methylation was determined based on the number of methylated cytosines divided by the total number of cytosines within the analyzed sequences from each PCR clone. Asterisks mark significant differences from the methylation level of Col-0 ($P \leq 0.05$, Generalized Linear Model with Poisson error distribution was used to deal with count data).

in a gene encoding a WD-40 repeat family protein; two others were in genes encoding proteins of unknown function. The remaining one of the four mutations was found in the gene encoding DRD1, an SNF2 domain-containing protein. This mutation was located at the 5′ end of the fourth exon of the gene labeled *AT2G16390* and causes a non-synonymous substitution (Fig. 1C). To assess whether this mutation corresponded to the phenotype of *boil5*, *boil5* was crossed with a disruptant for *AT2G16390* (designated *drd1-6*) obtained from the Arabidopsis Biological Resource Center (numbered CS69758, http://abrc.osu.edu/). The transcript level of *ONSEN* in F1 plants was significantly higher than that in the wild-type (Fig. 1D). These results strongly suggest that the causative factor of *boil5* is a mutation in the DRD1 gene.

DRD1 regulates the expression of a heat-activated *ONSEN* The heat shock transcription factor HsfA2 is an important factor among a subset of stress response genes and is required for *ONSEN* activation in plants subjected to heat stress (Cavrak et al., 2014). To reveal whether the amount of HsfA2 affected the expression level of *ONSEN* in the *drd1-6* mutant, we compared the transcript level of *HsfA2* between wild-type and *drd1-6* mutant. There was a significant increase of *ONSEN* transcript in *drd1-6* under heat stress conditions, although the transcript level of *HsfA2* was not significantly different (Fig. 3A and 3B). This observation revealed that the increase in transcript level of *ONSEN* in *drd1-6* was not correlated with the transcript level of *HsfA2*.

DRD1 regulates *ONSEN* expression by DNA methylation RdDM leads to methylation of cytosines in all sequence contexts: CG, CHG and CHH (where H corresponds to A, T or C). To understand the role of DRD1 in the epigenetic regulation of *ONSEN*, DNA methylation level was analyzed in the LTR promoter of the *ONSEN* copy (*At1g11265*) that shows the highest expression level upon heat stress (Cavrak et al., 2014). DNA methylation levels of this *ONSEN* LTR sequence showed a significant difference for non-CG methylation between the wild-type and the *drd1-6* mutant: 76% of CHG was methylated in the wild-type, compared to 35% in the *drd1-6* mutant. The methylation level of CHH was 7.1% in *drd1-6* compared to 60% in the wild-type. The *drd1-6* mutant showed the same hypomethylation state of non-CG as the *nrpd1-3* mutant (Fig. 4A and 4B).

Transgenerational transposition of *ONSEN* is not detected in heat-stressed *drd1-6* To detect transgenerational transposition of *ONSEN* in *drd1-6*, Southern blot analysis was performed in the offspring of *drd1-6* plants subjected to heat stress. No individuals having *ONSEN* transposition were found. In several individuals of that generation of *nrpd1-3*, which was used as a control, new *ONSEN* insertions were detected, suggesting that there are other important factors for *ONSEN* transposition besides *ONSEN* expression levels and DNA methylation levels on the *ONSEN* sequences (Fig. 5).

DISCUSSION

Here we report that a plant-specific SWI/SNF-like chromatin remodeling protein, DRD1, regulates the expression of a heat-activated retrotransposon. DRD1 was first identified in a screen for mutants impaired in RdDM (Kanno et al., 2004), and a subsequent study demonstrated that the endogenous targets of DRD1 were trans-

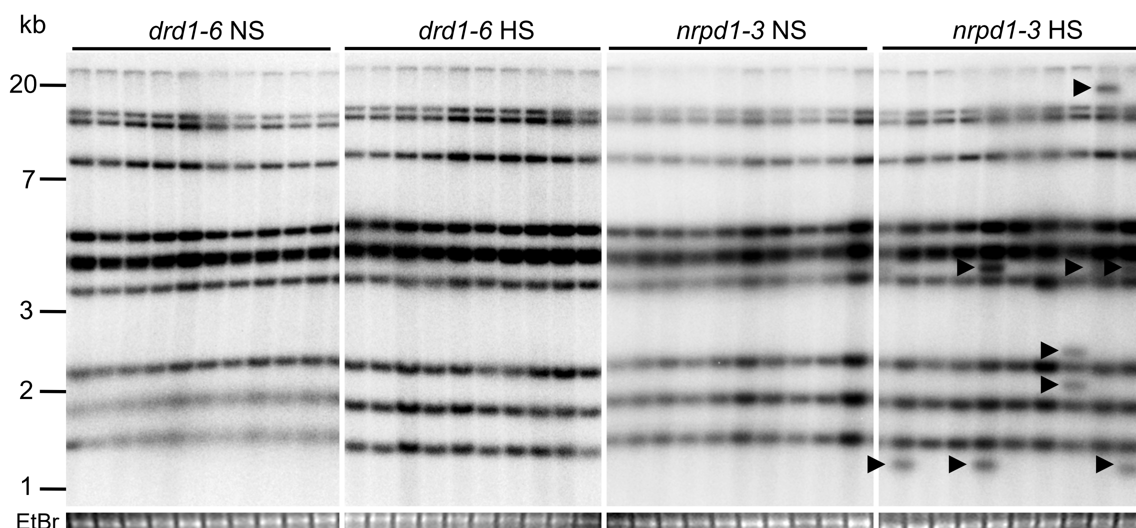

Fig. 5. Southern blot analysis of *ONSEN* in progeny of heat-stressed *drd1-6* and *nrpd1-3*. Each arrowhead indicates a new insertion of *ONSEN*. A gel stained with ethidium bromide (EtBr) is shown at the bottom of each panel as a loading control.

posons or sequences that encode short RNAs (Huettel et al., 2006). Compared with the well-studied SNF2-like protein DDM1, which regulates global DNA methylation, DRD1 acts locally to regulate levels of non-CG methylation (Jeddeloh et al., 1999; Kanno et al., 2004). Several heterochromatic repeats lose CG methylation in *ddm1* mutants but non-CG methylation in *drd1* mutants, suggesting that DRD1 is important for non-CG methylation of target sequences (Huettel et al., 2006). The sequence of the *ONSEN* LTR contains 78 cytosines in non-CG contexts that could be targets for DRD1-mediated RdDM. As expected, the levels of CHG and CHH methylation on the *ONSEN* sequence were significantly reduced in the *drd1* mutant compared to the wild-type.

Most transposons are silenced under non-stress conditions and are not activated in the *drd1* mutant. We found that the expression of heat-activated *ONSEN* was upregulated in the *drd1* mutant compared with the wild-type. The transcript level of *ONSEN* may be affected by chromatin state, which can be changed by heat stress. Although SWI2/SNF2 chromatin remodeling proteins play roles in stress responses (Shaked et al., 2006; Han et al., 2012; Gentry and Hennig, 2014), further research will be needed to determine the chromatin modification of *ONSEN* in the heat-stressed *drd1* mutant.

One possible mechanism to explain the upregulation of *ONSEN* in the *drd1* mutant subjected to heat stress is that the increase of *ONSEN* transcript is affected by the transcript level of *HsfA2* in the *drd1* mutant. Another possibility is that the physical association of HsfA2 with its targets is enhanced in the *drd1* mutant under heat stress. The latter mechanism is supported by the fact that the level of HsfA2 transcription was not significantly increased in the *drd1* mutant compared with the wild-type under heat stress conditions. However, we cannot exclude the possibility that a factor(s) other than the redundant heat-related transcription factor family is required for *ONSEN* activation under heat stress. The exact mechanisms underlying heat shock factor-mediated upregulation remain to be further elucidated.

The endogenous targets of DRD1 silencing machinery are short-RNA-encoding elements that are located in the 5′ flanking region of the target sequences (Huettel et al., 2006). In general, the target sequences that were upregulated in the *drd1* mutant had a euchromatic character and reside in gene-rich regions (Huettel et al., 2006). In the Col-0 accession, seven full-length *ONSEN* copies exist in euchromatic regions and one copy is located in centromeric heterochromatin. Further analysis may reveal whether the expression of each copy can be equally regulated by DRD1.

It is necessary to separate transcriptional activity from transposable activity when discussing the activation of transposons. An increase in the level of transcription is not necessarily associated with an increase in the frequency of transposition. As shown in our study, *ONSEN* is upregulated by heat stress in the *drd1* mutant to the same level as in the *nrpd1* mutant, but transgenerational transposition of *ONSEN* was not observed in the *drd1* mutant, whereas it was observed in the *nrpd1* mutant. To explain the difference, the properties of the *ONSEN* insertion sites need to be investigated, but at present, no common motifs have been found in the primary sequences of *ONSEN* insertion sites, and it is not clear what targets are used to determine the insertion sites. Previous studies, however, have shown that *ONSEN* tends to be inserted into euchromatin genes (Ito et al., 2016). Therefore, it is possible that DNA demethylation is induced in the *drd1* mutant, but chromatin condensation is maintained. This distinction may account for the lack of transgenerational transposition of *ONSEN* even though the element has similar methylation levels and increased expression in both the *drd1* and *nrpd1* mutants.

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