Genes Genet. Syst. (2016) **91**, p. 293–299

Characterization of a heat-activated retrotransposon in natural accessions of *Arabidopsis thaliana*

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(Received 14 August 2016, accepted 4 October 2016; J-STAGE Advance published date: 14 December 2016)

Natural accessions are used for studying intraspecies genetic variation in the model plant *Arabidopsis thaliana* in order to address fundamental questions of evolution. Transposable elements are responsible for a wide range of mutations and play significant roles in shaping a genome over evolutionary time. In the present study, we aimed to characterize *ONSEN*, a heat-activated long terminal repeat (LTR) retrotransposon, in natural *A. thaliana* accessions. Southern blot analysis demonstrated that *ONSEN* was present in all the studied accessions, but the copy number was diverse. Olympia-1 contained a single *ONSEN* copy, located in the centromere of Chromosome 3. A premature stop codon in Olympia-1 *ONSEN* presumably abolishes integrase activity, which in turn presumably renders the retrotransposon non-functional. Hybridization of Col-0 with Olympia-1 showed that several *ONSEN* copies in Col-0 were activated by heat stress and maintained their transpositional activity in the progeny.

Key words: *Arabidopsis*, heat stress, mutation, *ONSEN*, retrotransposon

INTRODUCTION

Arabidopsis thaliana has been used as a model organism in plant biology because of its relatively small genome and short life cycle. It is a predominantly self-pollinated plant and is widely distributed in the temperate zone (Maloof et al., 2001; Beck et al., 2008). More than 1,000 natural accessions have been isolated and characterized, revealing remarkable phenotypic variation in both morphological (e.g., leaf shape) and physiological (e.g., flowering time) traits (Ossowski et al., 2008; Atwell et al., 2010; Cao et al., 2011; Schneeberger et al., 2011). Natural genetic variation is considered to be partially influenced by changes in genome structure that are attributable to the abundance and distribution of transposable elements (TEs) (Madlung et al., 2012).

TEs cover a large portion of the genome in many plant species (e.g., more than 70% of the maize genome) and can affect gene expression when inserted near or within a gene-coding region (Sanmiguel and Bennetzen, 1998; Makarevitch et al., 2015). Previous studies have shown that TEs are regulated by epigenetic changes (DNA methylation or histone modification) and activated by environmental stress conditions (Chandler and Walbot, 1986; Bennetzen, 1987; Hirochika, 1993; Grandbastien et al., 1997; 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).

ONSEN is a heat-activated *Ty1*/*copia*-like retrotransposon in *A. thaliana* (Pecinka et al., 2010; Tittel-Elmer et al., 2010; Ito et al., 2011). In addition, *ONSEN*-related copies have been found in most species of the family Brassicaceae (Ito et al., 2013). Eight full-length *ONSEN* copies in the reference accession Columbia-0 (Col-0) are regulated by heat-shock factors (HSFs) that recognize heat-shock elements (HSEs) in heat-activated genes (Cavrak et al., 2014). Of 21 HSFs in *A. thaliana*, HsfA2 regulates the activation of *ONSEN*, which contains HSEs in the promoter regions of its long terminal repeats (LTRs) (Cavrak et al., 2014). Three of the eight copies contain identical LTRs, indicating their relatively recent transposition. An extrachromosomal DNA is synthesized from an expressed *ONSEN* copy following activation by heat stress (Ito et al., 2011). In Col-0, extrachromosomal DNAs are derived from six of the eight *ONSEN* copies (Cavrak et al., 2014), but their transpositional activity remains unknown.

A mutant deficient in small interfering RNA (siRNA) biogenesis showed higher transcript levels of *ONSEN*, while transposition was detected in the progeny of a heat-stressed mutant, indicating that the activation of *ONSEN*

Edited by Hidenori Nishihara
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DOI: http://doi.org/10.1266/ggs.16-00045

is controlled by siRNA-mediated epigenetic regulation (Ito et al., 2011). However, transcriptional activation and mobility of the eight Col-0 *ONSEN*s in a mutant background remain unclear. In the present study, we found that the accession Olympia-1 contains a single copy of *ONSEN*. Segregation of the Col-0 copies by crossing the siRNA mutant with Olympia-1 allowed us to characterize the transpositional activity of *ONSEN* copies subjected to heat stress.

MATERIALS AND METHODS

Plant material and growing conditions The set of 96 accessions (CS22660) used for Southern blot analysis was previously described by Nordborg et al. (2005). Four natural accessions, Olympia-1 (CS75905), Faner-1 (CS75670), Tanz-1 (CS75924) and Tanz-2 (CS75925), were added for Southern blotting. The *nrpd1a-3* mutant (Herr et al., 2005) was crossed with Olympia-1 for our hybrid analysis. The plants were grown on Murashige and Skoog (MS) plates at 21 °C under continuous light conditions.

Southern blot analysis Genomic DNA was isolated using the Nucleon PhytoPure DNA extraction kit (GE Healthcare Life Science, Chicago, IL, USA). Southern blotting was performed as described by Miura et al. (2004). Hybridization signals were detected using a radiolabeled *ONSEN*-specific probe (Supplementary Table S1), generated using the Megaprime DNA Labeling System (GE Healthcare Life Science). Genomic DNA of Col-0 was used as a template for the probe.

Heat stress treatment Seven-day-old seedlings grown on MS plates were subjected to a temperature shift from 21 °C to 37 °C for 24 h. Two days after this heat treatment, the seedlings were transplanted to two-inch pots with expanded vermiculite and allowed to grow at 21 °C.

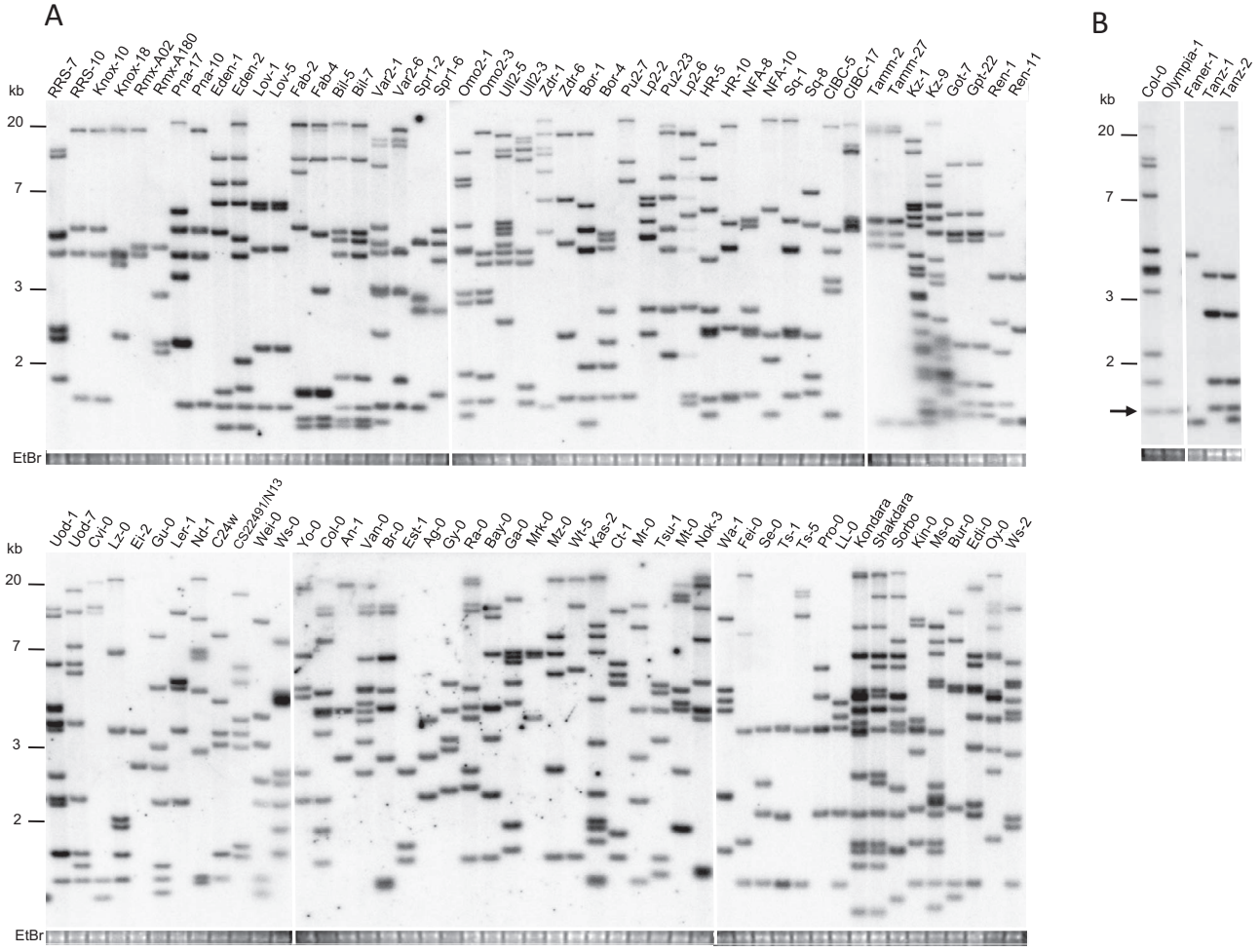

Fig. 1. Southern blots of *ONSEN* copies in 96 *Arabidopsis thaliana* accessions (A) and four additional natural *A. thaliana* accessions (Olympia-1, Faner-1, Tanz-1 and Tanz-2) with the reference accession Columbia-0 (Col-0) (B). Genomic DNA was digested with EcoRV and hybridized with an *ONSEN*-specific probe. The arrow in (B) indicates the conserved copy in Col-0 and Olympia-1. A gel stained with ethidium bromide (EtBr) is shown at the bottom of each panel as a loading control.

Real-time PCR Total RNA was extracted from whole seedlings grown on MS medium using TRI Reagent (Sigma Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. Approximately 3–5 μg of total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and reverse-transcribed using the ReverTraAce qPCR RT Kit (Toyobo, Osaka, Japan) with an oligo(dT) primer. To quantify the amount of *ONSEN* DNA, genomic DNA was extracted from leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Real-time PCR was performed using the Applied Biosystems 7300 Real Time PCR System with Thunderbird SYBR qPCR Mix (Toyobo). Three biological repetitions were performed, and standard deviation was calculated. DNA quantity was determined from a standard curve and normalized to the amount of 18S rDNA.

Sequence analysis A full-length *ONSEN* from the Olympia-1 genome was amplified by PCR. PCR primers (Supplementary Table S1) were designed to amplify *AT3G32415* from the Col-0 genome (TAIR10 Whole genome). The first half of *ONSEN* was amplified with AT3G32415full-F and Olympia-seq-R primer pairs and the latter half was amplified using Maseq-1751 and AT3G32415full-R primers. The PCR fragments were sequenced after cloning into pGEM-T Easy Vector (Promega) (Supplementary Fig. S1).

Phylogenetic analysis Phylogenetic relationships were analyzed using the neighbor-joining method, and Jukes-Cantor distances were calculated from full-length *ONSEN* sequences. Indel sites were treated with the complete deletion option. All analyses were performed with MEGA 6.0 (Tamura et al., 2013). Bootstrap probabilities were estimated by 500 replications.

RESULTS

Copy number of *ONSEN* in natural *A. thaliana* accessions To identify the genomic copy number diversity of *ONSEN*, we conducted a Southern blot analysis with an *ONSEN* probe in 100 *A. thaliana* accessions. The results showed that *ONSEN* was present in all accessions, while the copy number varied among the accessions (Fig. 1, A and B). A single *ONSEN* copy was detected in Olympia-1 (Fig. 1B).

Characterization of *ONSEN* in Olympia-1 PCR was conducted using a common internal primer within *ONSEN* (8copies-REVERSE) and a second primer in the flanking region that was specific for each copy of *ONSEN* in Col-0. The single *ONSEN* copy in Olympia-1 was conserved with the same flanking sequence as in Col-0 *AT3G32415* (Fig. 2A). Its sequence was 99.6% identical to *AT3G32415* (Supplementary Fig. S1) and phylogenetically closer to it than to other copies in Col-0 (Fig. 2B). Two of the predicted open reading frames of *AT3G32415* were non-functional owing to the presence of stop codons within the gene-coding regions (Fig. 2C). The *ONSEN* copy in Olympia-1 also contained a stop codon in the same position within the integrase gene-coding region in Col-0 *AT3G32415*, indicating its transpositional deficiency (Fig. 2C). The two LTRs located at both ends of *ONSEN* in Olympia-1 were 98% identical to those in Col-0 *AT3G32415*, indicating that this copy is relatively old, because young copies contain 100% identical LTRs such as *AT1G11265*, *AT3G61330* and *AT5G13205* in Col-0 (Ito et al., 2011).

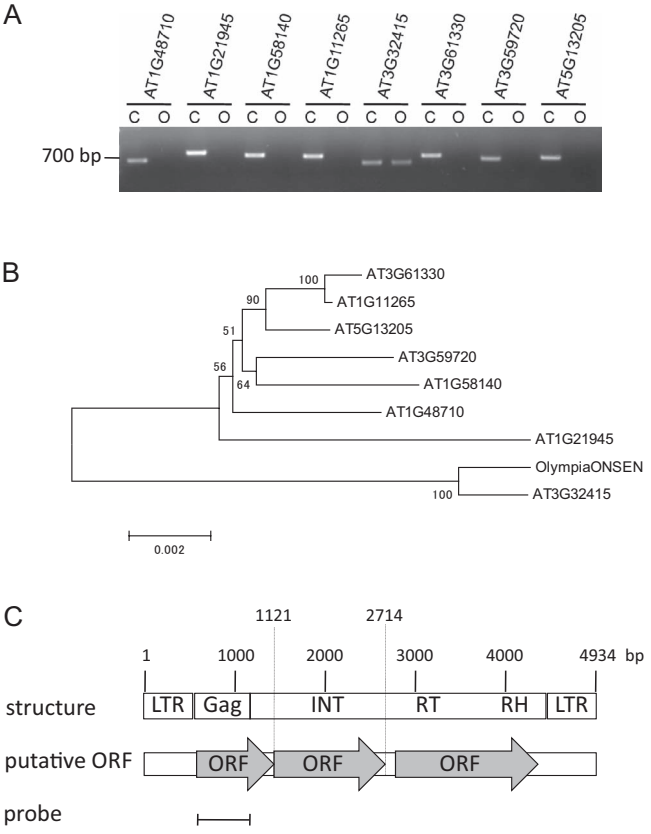

Fig. 2. Sequence analysis of *ONSEN* copies in *A. thaliana* Olympia-1 and Col-0. (A) PCR analysis for detecting the conserved copy of *ONSEN* in Col-0 (C) and Olympia-1 (O). (B) Neighbor-joining tree of *ONSEN* copies in Olympia-1 and Col-0. The single copy in Olympia-1 and the eight copies in Col-0 are labeled in Supplementary Fig. S1. Bootstrap probabilities are shown near the branches. A scale bar indicating genetic distance (JC distance) is shown at the bottom left side of the tree. (C) The structure of Col-0 *AT3G32415* and the *ONSEN* copy in Olympia-1. LTR: Long terminal repeat; Gag: Capsid protein; INT: Integrase; RT: Reverse transcriptase; RH: RNase H. Gray arrows mark open reading frames larger than 500 bp. Stop codons were found at the positions 1121 and 2714 bp. The region used as a probe for Southern blots is shown at the bottom.

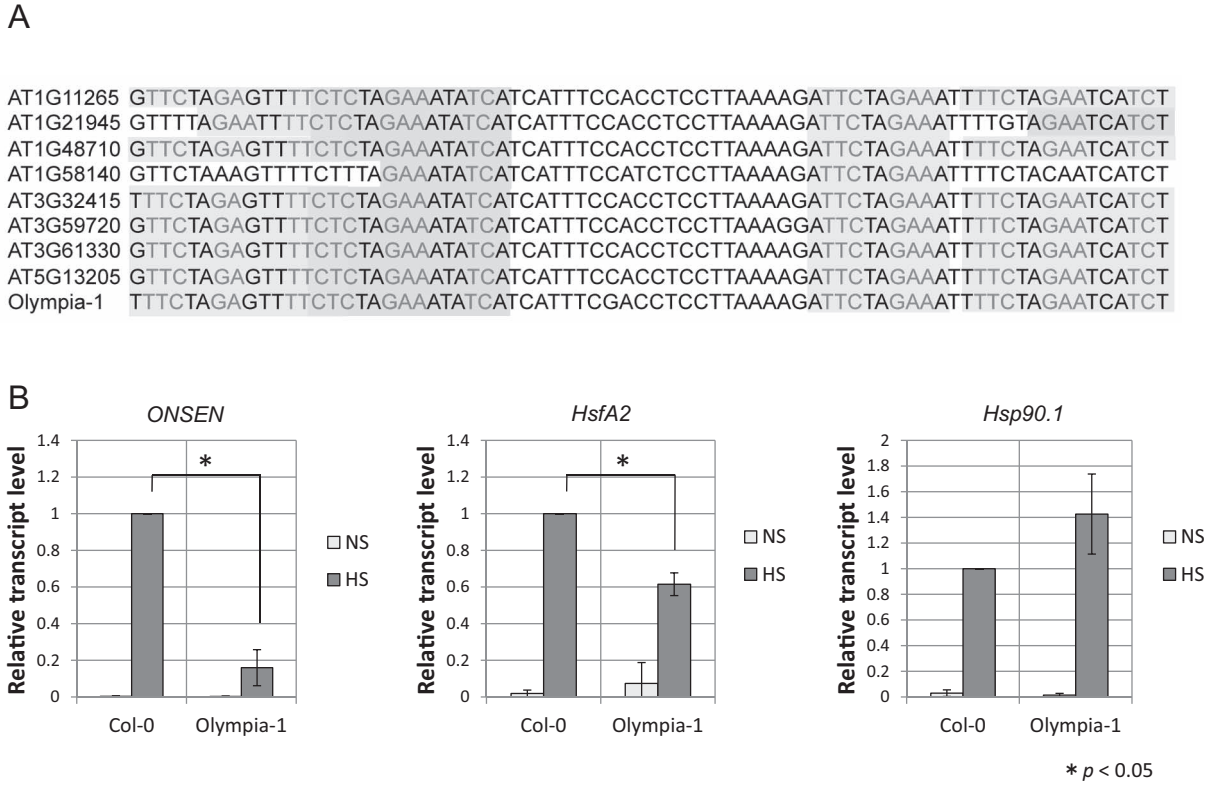

Fig. 3. Heat activation of *ONSEN*. (A) DNA alignment of the 81–156 bp section of LTRs in *A. thaliana* Olympia-1 and Col-0. Conserved heat shock elements (HSEs) (nTTCnnGAAnnTTCn or nGAAnnTTCnnGAAn) and possible HSEs (nGAAnnTTCor nTTCnnGAA) are highlighted. (B) Relative transcript levels of *ONSEN*, *HsfA2* and *Hsp90.1* in Olympia-1 and Col-0. Error bars represent the mean ± standard deviation (n = 3). Values are relative to heat-stressed Col-0. NS, non-stress; HS, heat stress. Relative transcript level of *ONSEN* was normalized by the copy number (Col-0:Olympia-1 = 8:1). The relative transcript levels of *ONSEN*, *HsfA2* and *Hsp90.1* in Col-0 were compared with the respective levels in Olympia-1 using a *t*-test. An asterisk indicates a significant difference, $P < 0.05$.

Heat activation of *ONSEN* in Olympia-1 To analyze heat activation of *ONSEN* in Olympia-1, we compared the sequences of HSEs in Olympia-1 and Col-0. HSEs were conserved in the eight *ONSEN* copies in Col-0 and the single copy in Olympia-1 (Fig. 3A). Heat activation of *ONSEN* was observed in Olympia-1; however, the transcript level of the single *ONSEN* copy in Olympia-1 was lower than that of the *ONSEN*s in Col-0, suggesting that the former was not fully activated or transcribed by heat stress (Fig. 3B). Additionally, the transcript level of two heat-responsive genes, *HsfA2* and *HSP90.1*, in Olympia-1 was up-regulated by heat stress, suggesting that HsfA2-mediated transcriptional activation can function in Olympia-1 (Fig. 3B).

Heat-induced transposition of *ONSEN* copies in Col-0/Olympia-1 To analyze the mobility of each *ONSEN* copy in Col-0, we crossed Olympia-1 with Col-0 (*nrpd1a-3*). The F_2 generation was genotyped for *nrpd1a-3* mutations, and the *ONSEN* copies present in F_2 genotypes were identified by PCR (Supplementary Table S1). *nrpd1a-3*-homozygous plants were heat-stressed, and their progeny were analyzed by Southern blotting. A new *ONSEN* copy was detected in an *nrpd1a-3*-homozygous Col-0/Olympia-1 progeny line that contained the *ONSEN* copies *AT1G11265*, *AT1G48710*, *AT3G61330* and *AT5G13205* (Fig. 4, A–C); however, no new *ONSEN* copies were detected in the progeny lines that contained only *AT1G58140* and *AT3G32415* (Fig. 4D). The results suggested that *AT3G61330*, *AT5G13205*, *AT1G11265* and/or *AT1G48710*, but not *AT1G58140* or *AT3G32415*, have transposition activity (Fig. 4B).

DISCUSSION

The copy number of some TEs differs among natural *A. thaliana* variants (Miura et al., 2004). Southern blot analysis showed that *ONSEN* was present in all the studied accessions, but the copy number in the genome varied among the accessions. The low frequency of each locus indicated that most copies cannot increase their frequency after transposition and are degraded. *ONSEN* was highly activated in the *nrpd1a-3* mutant, and transgenerational transposition was observed in the progeny (Ito et al., 2011). The copy number of *ONSEN* in the analyzed accessions was lower than that in the *nrpd1a-3* mutant

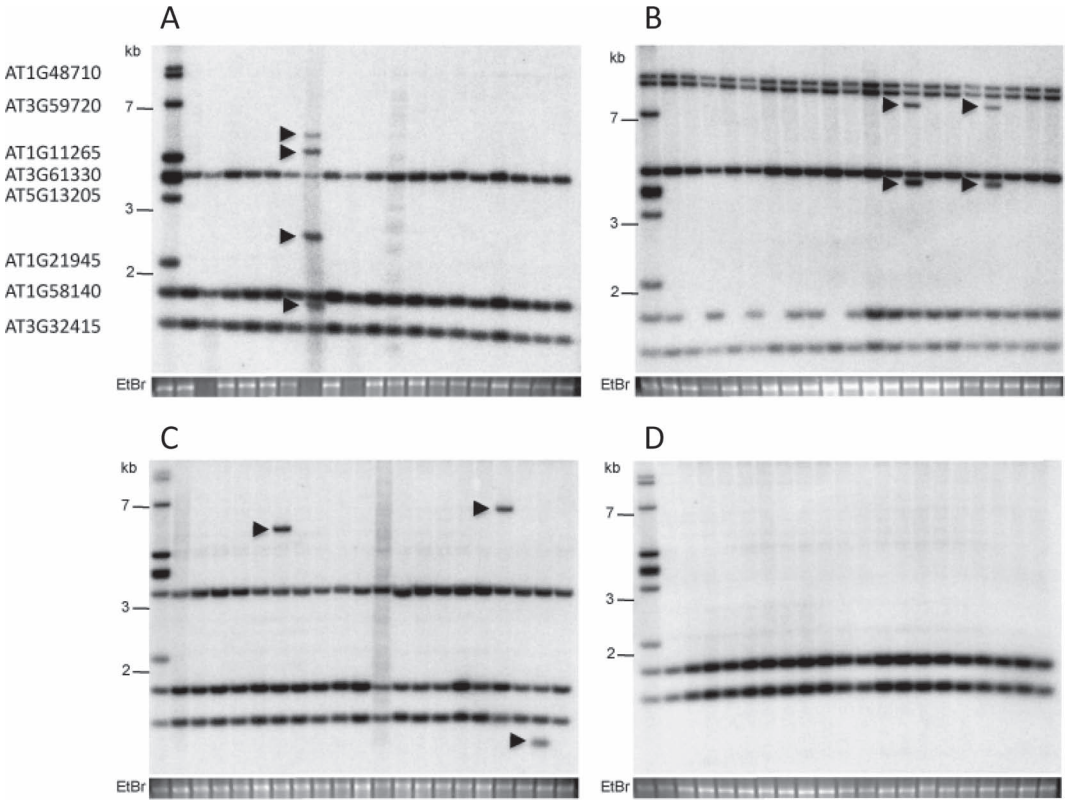

Fig. 4. Transpositional and transcriptional activation of progeny lines derived from heat-stressed *nrpd1*-homozygous F_2 Col-0/Olympia-1 plants. *AT3G32415* and the single copy of *ONSEN* derived from Olympia-1 are indistinguishable and are named *AT3G32415*. Arrowheads show new *ONSEN* copies detected by Southern blotting in progeny lines that contained *AT1G58140*, *AT3G32415* and *AT3G61330* (A), *AT1G11265, AT1G48710*, *AT1G58140* and *AT3G32415* (B), or *AT1G58140*, *AT3G32415* and *AT5G13205* (C). No new *ONSEN* copies were detected in progeny lines that contained *AT1G58140* and *AT3G32415* (D). Corresponding bands of each *ONSEN* copy are indicated on the left side of (A). The first lane in each panel is the Col-0 parent line. A gel stained with ethidium bromide (EtBr) is shown as a loading control at the bottom of each panel. The upper of the two bands marked as *AT1G48710* in (A) represents cross-hybridization of the probe with a genomic Col-0 DNA fragment that contains a region similar in sequence to *ONSEN*.

subjected to heat stress, suggesting that somatic transposition was tightly controlled by small RNA-mediated epigenetic regulation and did not pass into the progeny, although transcriptional activation was induced by heat stress.

The expression level of *ONSEN* in Olympia-1 was approximately 16% of that in Col-0. In Olympia-1, *ONSEN* is located in the centromere of Chromosome 3, in which constitutive heterochromatin suppresses gene expression, indicating that the expression of *ONSEN* is influenced by a positional effect. Previously, we reported that the *ONSEN* copy located in the centromere was highly conserved in 53 out of 95 studied *A. thaliana* accessions; however, it did not affect the gene expression of the host plant and escaped from natural selection (Ito et al., 2013). Therefore, it would be useful to analyze the transcript level of each *ONSEN* copy in Col-0, although distinguishing between their transcripts is difficult.

Multiple copies of *ONSEN* are found in most *A. thaliana* accessions. To identify the *ONSEN* copy that was autonomous in Col-0, we crossed Col-0 with Olympia-1, which contains only a single copy of *ONSEN*. Since transposition could be induced in a mutant that was deficient in small RNA-mediated regulation, we crossed the *nrpd1a-3* mutant (Col-0 background) with Olympia-1 to segregate copies of *ONSEN* in Col-0. Transposition of *ONSEN* was observed in several *nrpd1*-homozygous Col-0/Olympia-1 progeny lines that contained segregated *ONSEN* copies derived from Col-0. Three copies in Col-0 (*AT1G11265*, *AT3G61330* and *AT5G13205*) contained identical LTRs, indicating their recent transposition. The transposition of multiple *ONSEN* copies was consistent with a previous study, which revealed that the integrase-coding region of six *ONSEN* copies in Col-0 is conserved, suggesting that they are potentially transposable, although their transposition frequency is probably regulated epigenetically (Cavrak et al., 2014).

In the present study, the frequency of transgenerational transpositions of *ONSEN* derived from Col-0 in the Col-0/Olympia-1 hybrid was variable (5–16% with one active copy, Fig. 4), indicating that transposition occurred with different frequencies among the individual copies. It has

been reported that epigenetic modifications differ among *A. thaliana* accessions (Becker et al., 2011; Schmitz et al., 2013); therefore, it is worth considering that the transposition of *ONSEN* might be regulated by a hybrid-specific epigenetic modification. Genome-wide epigenetic information about Col-0 and Olympia-1 might allow us to obtain further detailed data on the underlying regulatory mechanisms.

We found that *ONSEN* was present in natural *A. thaliana* accessions, although the copy number was different among their genomes. Previous studies have reported an increase and decrease in the copy number of TEs in *A. thaliana*, as well as the selection of smaller genomes and the recent reduction of transposition, possibly owing to the transition to selfing (Hu et al., 2011; de la Chaux et al., 2012; Shimizu and Tsuchimatsu, 2015). *ONSEN* is regulated mainly by temperature, and the accumulation of multiple copies may have a deleterious effect on the host plants by destroying their gene function. Thus, the transposition–degradation cycle may determine the current *ONSEN* organization in natural *A. thaliana* accessions.

This study was supported by a Grant-in-Aid for Regional R&D Proposal-Based Program from the Northern Advancement Center for Science & Technology of Hokkaido, Japan, the NIG Collaborative Research Program (2015-A1 18), JST-PRESTO, a Grant-in-Aid for JSPS Fellows (14J02452), a Grant-in-Aid for Scientific Research on Innovative Areas (JP15H05960), and a Grant-in-Aid for Scientific Research in Innovative Areas (2511970103).

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