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Inducible transposition of a heat-activated retrotransposon in tissue culture

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Running head: Transposition of *ONSEN* in tissue culture

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19 **Inducible Transposition of a Heat-activated Retrotransposon in Tissue Culture**

20 Running head: Transposition of *ONSEN* in tissue culture

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30

31 **Abbreviations**

32 ABA; abscisic acid, AGO4; ARGONAUTE 4, DCL3; DICER-LIKE 3, DRM2; DOMAINS
33 REARRANGED METHYLTRANSFERASE 2, IBM2; INCREASE IN BONSAI METHYLATION2,
34 LTR; long terminal repeat, PolIV; RNA polymerase IV, PolV; RNA polymerase V, RdDM; RNA-directed
35 DNA methylation, RDR2; RNA-dependent RNA polymerase 2, suvh2; Suppressor of variegation 3-9
36 homolog protein2, TEs; transposable elements.

37

38 **Footnotes**

39 The read data were submitted to the DDBJ Read Archive (Accession number DRA003007 and
40 DRA004828).

41

Abstract

A transposition of a heat-activated retrotransposon named *ONSEN* required compromise of a small RNA-mediated epigenetic regulation that includes RNA-directed DNA methylation (RdDM) machinery after heat treatment. In the current study, we analyzed the transcriptional and transpositional activation of *ONSEN* to better understand the underlying molecular mechanism involved in the maintenance and/or induction of transposon activation in plant tissue culture. We found the transposition of heat-primed *ONSEN* during tissue culture independent to RdDM mutation. The heat activation of *ONSEN* transcripts was not significantly upregulated in tissue culture compared with that in heat-stressed seedlings, indicating that the transposition of *ONSEN* was regulated independently of the transcript level. RdDM-related genes were upregulated by heat stress both in tissue culture and seedlings. The level of DNA methylation of *ONSEN* did not show any change in tissue culture, and the amount of *ONSEN*-derived small RNAs was not affected by heat stress. The results indicated that the transposition of *ONSEN* was regulated by an alternative mechanism in addition to the RdDM-mediated epigenetic regulation in tissue culture. We applied the tissue culture-induced transposition of *ONSEN* to Japanese radish, an important breeding species of the family Brassicaceae. Several new insertions were detected in a regenerated plant derived from heat-stressed tissues and its self-fertilized progeny, revealing the possibility of the molecular breeding without genetic modification.

Keywords

Brassicaceae, heat stress, *ONSEN,* tissue culture, transposon

Introduction

Plant cells retain totipotency and developmental plasticity in diverse tissues. Plants have the ability to form dedifferentiated cells, called callus, that subsequently regenerate into mature plants under appropriate culture conditions in response to hormonal stimuli (Skoog and Miller 1957; Steward et al. 1964). During tissue culture, some genetic and epigenetic changes may occur in callus (Jiang et al. 2011; Jiang et al. 2015; Kaeppler et al. 2000a, b; Neelakandan and Wang 2012). Mutations, such as cytogenetic abnormalities, sequence changes, and transposon movements, occur in high frequency in plants regenerated from undifferentiated cell cultures, inducing multiple genetic changes (Johnson et al. 1987; Sato et al. 2011a). Callus-induced genetic modifications also include changes in DNA methylation (Berdasco et al. 2008; Brown et al. 1991; Brown et al. 1990; Loschiavo et al. 1989; Shemer et al. 2015). Studies on the global methylation levels and methylation of specific sites have shown that variation in DNA methylation frequently occurs in tissue culture.

Callus has been identified as the tissue with the highest number of transposable elements (TEs). The expression and transpositional activation of TEs have been demonstrated in callus-regenerated plants (de Araujo et al. 2005; Hirochika 1993b; Hirochika et al. 1996; Madsen et al. 2005; Planckaert and Walbot 1989; Sato et al. 2011b; Yamashita and Tahara 2006; Yilmaz et al. 2014). The activation of quiescent transposons indicates that epigenetic changes occur through the culture process. In *Arabidopsis thaliana,* the activation of TEs is under epigenetic control, called RNA-directed DNA methylation

(RdDM). An ectopic transcript produced from TEs by a DNA-dependent RNA polymerase, known as RNA polymerase IV (PolIV), is transcribed to double-stranded RNA by RNA-dependent RNA polymerase 2 (RDR2) and produces double-stranded precursors that are processed by DICER-LIKE 3 (DCL3) and generate 24-nt to 26-nt siRNAs (Kanno et al. 2005; Mosher et al. 2008; Pontier et al. 2005; Zhang et al. 2007). The siRNAs bind to an RNA-induced silencing complex that contains ARGONAUTE 4 (AGO4), interact with RNA polymerase V (PolV) to recruit the DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), and cause the *de novo* DNA methylation of the target TEs (Cao and Jacobsen 2002; Matzke and Birchler 2005).

Environmental stress alters the chromatin structure that is associated with epigenetic processes such as histone modification and DNA methylation. Although most TEs are silenced due to epigenetic modifications, environmental changes may induce transposon activation (Grandbastien 2004). Focusing on environmental stress as a trigger for the activation of TEs, we found the heat-stressed activation of a *Ty1*/*Copia*-like retrotransposon, named *ONSEN*, in *A. thaliana* (Ito et al. 2011). The activated *ONSEN* is not only transcribed, but also transposed in heat-stressed plants that are deficient in the small RNA-mediated RdDM pathway. In *A. thaliana*, *ONSEN* is preferentially inserted within or close to genes (Ito et al. 2011) and changes the expression of flanking genes. For instance, the transposition of *ONSEN* generated a mutation in an abscisic acid (ABA) responsive gene, resulting in an ABA-insensitive phenotype in *A. thaliana* (Ito et al. 2016).

The molecular mechanism involved in the maintenance and/or induction of transposon activation during tissue culture in plants is still poorly understood. Here, we analyzed the transcriptional and transpositional activation of *ONSEN* in tissue culture. We found new *ONSEN* insertions in the regenerated *A. thaliana* plants without mutation in the RdDM machinery. The results might indicate the existence of an unknown mechanism that regulates *ONSEN* transposition.

Previously, we reported the presence of *ONSEN*-related copies in most species of the family Brassicaceae (Ito et al. 2013) as well as the heat-induced transcriptional activation of *ONSEN*, which indicates the presence of a conserved heat-responsive cis-element (Cavrak et al. 2014). Although most Brassicaceae species have *ONSEN* sequences, transposition has not been observed. In the current study, we applied the callus-mediated transposition of *ONSEN* in Japanese radish (*Raphanus sativus*), an important breeding species of the family Brassicaceae. This is the first report on the successful transposition of a heat-activated transposon in crops.

Results

Detection of new ONSEN insertions in tissue culture

To analyze the transpositional activation of *ONSEN* in tissue culture, callus was induced from the hypocotyl and cotyledon of a young heat-stressed *A. thaliana* seedling (Figure 1a–f) as shown in Figure 1j. As a control, callus was induced from the hypocotyl and cotyledon of a non-stressed seedling. The callus

was subsequently transferred to an appropriate organ induction medium. New *ONSEN* insertions were detected in the regenerated plant derived from the heat-stressed hypocotyl and cotyledon (Figure 2a, b). Transgenerational transpositions were also detected in the self-fertilized progeny; however, no new insertions were detected in the non-stressed regenerated plants and their progenies. To test whether the *ONSEN* transposition was induced in heat-stressed dedifferentiated cells, we analyzed the callus induced from a mature seed (Figure 1g–i). The seed-derived callus was exposed at 37ºC for 24 h and subsequently regenerated at 21°C (Figure 1j). New *ONSEN* insertions were detected in the regenerated plant and the self-fertilized progeny (Figures 2c). No new *ONSEN* insertions were detected in the F_2 generation, indicating that the activation of *ONSEN* was silenced in the F_1 generation without heat stress (Figures S1). Next, we analyzed a retrotransposition of *ONSEN* in callus derived from a heat-stressed seedling and a seed of the *NRPD1* mutant (*nrpd1*) that lacks functional PolIV, in which *ONSEN* is highly expressed. A large number of new insertions were detected in the regenerated plants and their self-fertilized progenies (Figure 2d–f).

Mapping of new insertions

To identify new *ONSEN* insertions, we carried out a genome-wide analysis of the self-fertilized progenies of wild-type and *nrpd1* plants regenerated from seed-derived callus. In wild-type, new insertions were identified in 23 loci (Table S1); 20 were located within genes (Figure 3a), and 15 of these within exons. In

nrpd1, new insertions were identified in 152 loci (Table S2); 120 were located within genes (Figure S2), and 100 of these within exons. The results indicated that the new *ONSEN* copy could target genes in euchromatic regions both in the wild type and *nrpd1*.

The effect of new ONSEN insertions in a regenerated plant

To understand the effect of new *ONSEN* insertions on a host plant, we conducted a genome-wide microarray analysis of gene expression (37,251 genes in total) in the progeny of a regenerated *ONSEN*-inserted line growing at 21°C and identified 198 up-regulated and 504 down-regulated genes (Student's *t*-test at $P < 0.05$, Table S5, and S6). About 30% of down-regulated genes in the *ONSEN*-inserted line were categorized to intrinsic component of membrane (Table S7), although the number of differentially expressed genes based on the q values was much smaller (Table S5, and S6). Most genes that *ONSEN* inserted do not exhibit differential expression compared with wild type (Fig 3b). The results indicated that the alteration of gene expression was mainly caused by callus induction, and the effect was inherited to the next generation of a regenerated plant.

Expression profile of RdDM-related genes in callus

Previously, we reported a transgenerational transposition of *ONSEN* in heat-stressed mutants that are deficient in the small RNA-mediated RdDM pathway (Ito et al. 2011). To understand the epigenetic

regulation in callus, we compared the transcript level of genes that are related to the small RNA-mediated RdDM pathway in callus and seedlings. The related genes, including *NRPD1*, *RDR2*, *AGO4*, *DRM2*, and *NRPE1* (NRPE1 is a subunit of PolV), were significantly upregulated without heat stress in callus. Additionally, the transcript level of *NRPD1*, *RDR2*, *DCL3*, and *NRPE1* in callus was significantly higher than that in seedlings two weeks after the heat stress (Figure 4). Most of the genes had similar or higher expression levels in callus compared with those in seedlings, indicating that the RdDM pathway is functionally active in heat-stressed callus.

Detection of small RNA in callus

Since the transpositional activation of *ONSEN* is regulated by an siRNA-related pathway in seedlings (Ito et al. 2011), we analyzed the amount of small RNAs in wild-type seed-derived callus and also in wild-type seedlings using small RNA sequencing (sRNA-Seq). The level of siRNA derived from *ONSEN* (*AT5G13205* or transposon name *AT5TE15240*) was increased both in callus and seedlings (Figure 5a), whereas the amount of 24-nt siRNA was 10-fold higher and highly enriched in callus compared with that in seedlings 2 weeks after the heat stress (Figure 5b). In callus, the 24-nt siRNAs were mapped throughout the entire region of *ONSEN* 2 weeks after the heat stress. The high amount of *ONSEN*-derived 24-nt siRNA in callus indicated that the transpositional regulation of *ONSEN* was not affected by the siRNA accumulation.

172

173 *Activation of a heat shock factor and ONSEN in tissue culture*

174 The transcriptional activation of *ONSEN* is regulated by the heat shock factor, HsfA2 (Cavrak et al. 2014).

175 To understand the relationship between the expression of *HsfA2* and the activity of *ONSEN* in callus, we

176 analyzed their transcript levels in wild-type and *nrpd1* seed-derived callus and also in wild-type seedlings

177 immediately after the heat stress and 2 weeks after the heat stress. The transcript level of *HsfA2* was not

178 significantly upregulated in callus compared with that in seedlings (Figure 6a). The transcript level of

179 *ONSEN* was significantly upregulated in *nrpd1* seed-derived callus compared with that in wild-type

180 immediately after the heat stress, but not two weeks after the heat stress (Figure 6b). The results suggest

181 that the expression of *HsfA2* was not affected under the experimental conditions used in this study, and

182 that an RdDM-related pathway regulated the transcriptional activation of *ONSEN* in callus, similar as in

183 seedlings.

184

185 *DNA methylation of ONSEN in tissue culture*

186 DNA methylation is known to decrease or inhibit the transpositional activity of various transposable

187 elements. In order to test the effects of DNA methylation on the transpositional regulation of *ONSEN*, we

188 performed Southern blotting using methyl-sensitive restriction enzymes. The results showed that the DNA

189 methylation of *ONSEN* in callus was not affected by heat stress (Figure 6c), indicating that the release of

190 *ONSEN* transposition in heat-stressed tissue culture was probably independent of DNA methylation.

191

192 *Transposition of ONSEN in Japanese radish*

193 We analyzed the transposition of *ONSEN* in Japanese radish. Because of the variation of *ONSEN* copies

194 in the F_1 generation, we selected several Japanese radish lines with a fixed *ONSEN* copy. Callus was

195 induced from mature seeds (Figure 7a–h). The seed-derived callus was exposed at 37ºC for 24 h, and

196 subsequently, differentiation was induced at 21°C. New *ONSEN* insertions were detected in a regenerated

197 plant and the self-fertilized progeny (Figure 7i).

198

199 **Discussion**

200 This is the first report on the heat-activated retrotransposition of *ONSEN* in tissue culture. Heat stress is

201 necessary for the transpositional activation of *ONSEN* in tissue culture that is distinct from the activation

202 of some long terminal repeat (LTR) retrotransposons, including *Tnt1* and *Tto1* in tobacco (Hirochika

203 1993a, b) and *Tos17* in rice (Hirochika et al. 1996). In the present study, a heat-shock transcriptional

204 factors and the RdDM-mediated pathway could regulate the transcription and transposition of *ONSEN*

205 subjected to heat stress. The transposition of *ONSEN* was observed in a regenerated plant that was derived

206 from heat-stressed seedlings, indicating that the dedifferentiation and subsequent regeneration of

207 heat-stressed tissues could release the transpositional regulation of *ONSEN*. A somatic transposition might

occur in a differentiated cell of a heat-stressed seedling, and the subsequent culture might promote the dedifferentiation and cell proliferation of tissue with a new *ONSEN* insertion. The transposition of *ONSEN* was also observed in a regenerated plant that was derived from seed-derived callus, indicating that *ONSEN* could transpose in a heat-stressed dedifferentiated tissues and the following regenerated tissues. The insertion patterns of *ONSEN* were similar among the regenerated plants derived from a single callus, suggesting that the transposition occurred before the regeneration.

The transposition of TEs might change the gene expression of the inserted regions. Genome-wide analysis of the *ONSEN*-inserted progeny showed that the expression of *ONSEN*-integrated genes was slightly changed. The small effect of *ONSEN* insertion might be due to the low transcript level of the target genes or the ectopic transcription induced by the insertion, and compensate the expression of the mutated gene. A previous study, which showed that *ONSEN* insertions confer heat responsiveness to nearby genes, supports this assumption (Ito et al. 2011). In *A. thaliana,* the splicing of the TE-containing intron is promoted by the nuclear protein INCREASE IN BONSAI METHYLATION2 (IBM2) (Saze et al. 2013). Previously, we showed that IBM2 controls a new insertion of activated *ONSEN* copies embedded in the intron of an ABA-responsive gene (Ito et al. 2016). In the present study, some intronic insertions within transcribed genes might be marked by repressive epigenetic modifications induced by IBM2.

The activation of *ONSEN* in seedlings is induced by heat stress; however, no transposition is detectable in wild-type plants and their self-fertilized progenies (Ito et al. 2011), whereas *ONSEN* is

highly activated in *nrpd1* plants, and transgenerational transposition is observed in their progenies (Ito et al. 2011). Therefore, transposition is regulated by a small RNA-mediated pathway. In the present study, the somatic transposition of *ONSEN* was observed in a callus-derived regenerated plant. The frequency of transposition in wild-type callus was lower than that in *nrpd1* callus, indicating that transposition might be regulated by multiple factors involved in gene expression and epigenetic modification during explant dedifferentiation and redifferentiation (Neelakandan and Wang 2012). The fate of cultured cell is controlled by transient changes in gene expression (Berdasco et al. 2008), which is involved in the synthesis of hormones (e.g., cytokinin and auxin) and transcriptional regulators that affect development (Yin et al. 2008). To test the possibility that the callus-mediated transposition of *ONSEN* was regulated by the contiguous activation of *ONSEN*, we analyzed the expression of *HsfA2* in seedlings and callus. The transcript level of *HsfA2* was not significantly different between seedlings and callus. Additionally, the expression of *HsfA2* was transient and not maintained longer in callus compared with that in seedlings, indicating that the transposition of *ONSEN* might be regulated in a callus-specific manner. We previously reported that the transcript and extrachromosomal DNA of *ONSEN* were detected in callus 30 d after the heat stress (Matsunaga et al. 2012). The long-term and tissue-specific regulation of *ONSEN* activation in callus as well as the transcript level of *ONSEN* might affect the transposition frequency in tissue culture. The transcript level of *ONSEN* in wild-type callus was not significantly upregulated compared with that in wild-type seedlings, suggesting that the transposition of *ONSEN* was independent of the transcript level.

A previous study showed that the high amount of transcript of *ONSEN* in a histone methyltransferase mutant (*suvh2*) was not sufficient to induce the transposition of *ONSEN* (Ito et al. 2011).

The induction of *ONSEN* transposition in tissue culture could be related to open chromatin. In rice, the genome-wide mapping of DNase I hypersensitive (DH) sites in seedlings and callus revealed significant differences in the number and the total length of DH site-related DNA sequences between the two tissues (Zhang et al. 2012). The number of DH sites in callus was 58% higher than that in seedlings. These results suggested that open chromatin might be abundant in *A. thaliana* callus, allowing the transposition of *ONSEN* in tissue culture. Therefore, our results might suggest that open chromatin allowed the transposition of *ONSEN* in euchromatic regions.

Tissue culture-induced changes occur at the genome level and involve the epigenetic reprogramming of gene expression (Kaeppler et al. 2000b). Previously, we showed that the transposition of *ONSEN* was regulated by the RdDM-mediated pathway in wild-type plants (Ito et al. 2011). Therefore, we analyzed the expression level of RdDM-related genes in callus. The expression of these genes was not suppressed in callus, whereas some of them were upregulated. These results indicated that the RdDM pathway is functionally active in callus. Global methylation studies support the idea that developmental timing may affect the methylation levels and patterns in callus and regenerated plants (Huang et al. 2012; Li et al. 2011; Vining et al. 2013). DNA methylation patterns are highly variable among regenerated plants and their progenies, suggesting that their DNA modifications are less stable compared with those in

seed-grown plants (Kaeppler et al. 2000b). A transient downregulation of the RdDM-related pathway might activate the transposition of *ONSEN* in callus. To test this hypothesis, we analyzed the DNA methylation of *ONSEN* and the *ONSEN*-derived small RNAs. In callus, no significant change in the level of DNA methylation was observed before and after the heat stress, indicating that RdDM was functionally active in callus, although a transpositional regulation might be eased in tissue culture-specific regeneration. The accumulation of *ONSEN*-derived small RNAs in callus also supported the assumption that RdDM was functionally active in tissue culture. The amount of 24-nt siRNAs derived from heat-activated *ONSEN* was 10-fold higher in callus than in seedlings, results that were consistent with the upregulation of some RdDM-related genes in heat-stressed callus. The large number of 24-nt siRNAs was distributed in the entire region of *ONSEN*, explaining the *ONSEN*-wide establishment of RdDM in heat-stressed callus.

In crop species, the controlled retrotransposition of divergently regulated elements may generate individuals with novel variation in response to particular environmental stresses (Paszkowski 2015). Therefore, it is important to consider the self-regulating nature of retrotransposons under environmental and epigenetic conditions that leads to the genome-wide redistribution of regulatory signals due to proliferation and movement (Lisch 2013). The new insertions induce loss-of-function mutations that create new promoters, enhancers, or silencers, and consequently create new regulatory capabilities (Grandbastien 2015). Here, we introduced the new heat-activated *ONSEN* insertions in

Japanese radish. The response of *ONSEN* to particular environmental stimuli could extend the response of new genes and chromosomal areas that would dispatch selected regulatory elements throughout the genome. The novel distribution of *ONSEN* insertions in plant populations could produce a huge variety of regulatory networks responsive to environmental stimuli that would initiate retrotransposition. Therefore, the sequential and controlled mobilization of stress-activated retrotransposons within sizable populations may yield individuals with beneficial traits for plant breeding.

Material and Methods

Plant materials and growing conditions

We used wild-type and *nrpd1 A. thaliana* plants of the ecotype Columbia (Col-0). The seedlings were grown on Murashige and Skoog medium (MS; Sigma-Aldrich, St. Louis, MO, USA) under continuous light at 21°C. The seeds of Japanese radish (brand name YR Tengu) were obtained from Takii and Company (Kyoto, Japan).

Callus induction and heat stress

The leaves, hypocotyl, and cotyledon of 7-d-old *A. thaliana* seedlings were separated and subjected to a temperature shift from 21°C to 37°C for 24 h and immediately transferred to callus induction medium (CIM), containing 3.1 g L^{-1} Gamborg's B5 (Sigma-Aldrich) with 0.5 g L^{-1} MES, 20 g L^{-1} glucose, 2.5 g

298 L^{-1} gellan gum, 0.05 mg L^{-1} kinetin, and 0.5 mg L^{-1} 2,4-D. After 5–7-d of incubation at 21°C, the callus
299 was transferred to shoot induction medium (SIM), containing 3.1 g L^{-1} Gamborg's B5 with 0.5 g L^{-1} MES,
300 20 g L^{-1} glucose, 2.5 g L^{-1} gellan gum, 5 mg L^{-1} 2ip, and 0.1 mg L^{-1} indole-3-acetic acid (IAA). The
301 induced shoot was subsequently transferred to root induction medium (RIM), containing 4.3 g L^{-1} MS
302 with 0.5 g L^{-1} MES, 10 g L^{-1} sucrose, 2.5 g L^{-1} gellan gum, 2 mg L^{-1} 1-napthaleneacetic acid (NAA), and
303 1 ml L^{-1} 1,000× B5. For the heat-activation of *ONSEN* in callus, seeds were plated on CIM for two weeks,
304 and the induced callus was subjected to a temperature shift from 21°C to 37°C for 24 h. Two weeks later,
305 the heat-stressed callus was transferred to SIM. Next, the induced shoot was transferred to RIM. The
306 regenerated plants were transplanted to soil and allowed to grow at 21°C.
307 In Japanese radish, seeds were plated on pre-culture medium, containing 4.3 g L^{-1} MS with 0.5
308 g L^{-1} MES, 30 g L^{-1} sucrose, 2.5 g L^{-1} gellan gum, 1 mg L^{-1} 2,4-D, and 1 ml L^{-1} 1,000× B5, for 1 week.
309 Next, they transferred to CIM1, containing 4.3 g L^{-1} MS with 0.5 g L^{-1} MES, 10 g L^{-1} sucrose, 2.5 g L^{-1}
310 gellan gum, 3 mg L^{-1} BA, 1 mg L^{-1} zeatin, 3 mg L^{-1} silver nitrite, and 1 ml L^{-1} 1,000× B5, for two weeks
311 and then to CIM2, containing 4.3 g L^{-1} MS with 0.5 g L^{-1} MES, 10 g L^{-1} sucrose, 2.5 g L^{-1} gellan gum, 3
312 mg L^{-1} BA, 1 mg L^{-1} zeatin, and 1 ml L^{-1} 1,000× B5. Two weeks later, the induced calli were subjected to
313 a temperature shift from 21°C to 37°C for 24 h. Three weeks later, the heat-stressed callus was transferred
314 to SIM for radish, containing 3.1 g L^{-1} Gamborg's B5 with 0.5 g L^{-1} MES, 10 g L^{-1} sucrose, 2.5 g L^{-1}
315 gellan gum, 3 mg L^{-1} BA, 1 mg L^{-1} zeatin, and 1 ml L^{-1} 1,000× B5. Next, the induced shoot was

316 transferred to RIM for radish, containing 3.1 g L^{-1} Gamborg's B5 with 0.5 g L^{-1} MES, 10 g L^{-1} sucrose,
317 2.5 g L^{-1} gellan gum, and 1 ml L^{-1} 1,000× B5. The regenerated plants were transplanted to soil and
318 allowed to grow at 21°C. Callus induction and regeneration were conducted under continuous light
319 conditions.
320
321 *Detection of ONSEN insertion by whole genome sequencing*
322 Whole genome sequencing was conducted by HiSeq2500 (Illumina, San Diego, CA, USA). The sequence
323 reads in which > 10% of base calls had a Phred quality score < 30 were excluded from the subsequent
324 analysis. To identify the insertion positions of *ONSEN*, we first selected paired-end sequence reads that at
325 least one pair had 25–75% nucleotides corresponding to the upstream region of *ONSEN*. Then, we
326 trimmed off the nucleotides corresponding to *ONSEN* from the selected sequence reads. The processed
327 sequence reads were aligned to the *A. thaliana* reference sequence TAIR10 (http://arabidopsis.org) using
328 BWA (Li and Durbin, 2009). Alignment files were converted to SAM/BAM files using SAMtools (Li *et*
329 *al*., 2009), and the positions with enough depth at the border regions of the trimmed sequence reads were
330 defined as positions that *ONSEN* was inserted. The read data were submitted to the DDBJ Read Archive
331 (Accession number DRA003007).
332
333 *Microarray analysis*

Microarray experiments were performed using an Agilent DNA Microarray Scanner G2565BA with Feature Extraction v. 9.5.3 (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's protocol. Three biological replicates were used in each experiment. The quality of total RNA was performed by the Agilent 2100 Bioanalyzer system, and its integrity number (RIN) was > 8.0. cRNA was labeled using the Low Input Quick Amp Labeling Kit (Agilent) and hybridized to an Agilent custom array platform (Design ID = 034592). cRNA probes were designed based on the expression regions of the TAIR10 genome using previous tiling-array and RNA-Seq analyses (Kawaguchi et al. 2012; Matsui et al. 2008; Okamoto et al. 2010). We normalized the signals of microarray probes to the 75th percentile and set the criteria of Student's *t*-test at $P < 0.05$ to determine significance in differential gene expression.

Southern blot analysis

Plant genomic DNA was isolated using the Nucleon PhytoPure (GE Healthcare Life Science, Little Chalfont, UK). 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 radio-labeled *ONSEN*-specific probe (Table S8) that was generated with the Megaprime DNA Labeling System (GE Healthcare Life Science).

352 *Quantitative real-time PCR (qRT-PCR)*

353 Total RNA was extracted from 50 seedlings using the TRI Reagent (Sigma-Aldrich), following the
354 manufacturer's protocol. Approximately 3–5 μg of total RNA was treated with RQ1 RNase-free DNase
355 (Promega, Madison, WI, USA) and reverse-transcribed using the ReverTraAce qPCR RT Kit (Toyobo,
356 Tokyo, Japan). qRT-PCR was performed using the Applied Biosystems 7300 Real Time PCR System
357 (Applied Biosystems, Carlsbad, CA, USA) with the Thunderbird SYBR qPCR Mix (Toyobo). The
358 primers used for qRT-PCR are listed in Table S8. We performed three biological replicates and
359 determined the standard deviation of the replicates.

360

361 *Small RNA sequence analysis*

362 Total RNA was isolated from young seedlings and seed-derived callus. The quality of total RNA and the
363 quantity of small RNA were confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies).
364 Library preparation was performed using the TruSeq Small RNA Library Prep Kit (Illumina), according
365 to the manufacturer's protocol. Total RNA (1 μg), including small RNA, was connected with 3'-adapter
366 and 5'-adapter. The adapter-ligated RNA was reverse-transcribed and amplified with adapter-specific
367 primers. The amplified cDNA was fractionated from 145-bp to 160-bp using a 6% Novex TBE gel (Life
368 Technologies, Carlsbad, CA, USA). The size-selected cDNA was cleaned up using AMPure XP magnetic
369 beads (Beckman Coulter, Brea, CA, USA). Library quality and concentration were assessed using the

Agilent Bioanalyzer 2100 (Agilent Technologies) with the KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, MA, USA). The libraries were sequenced using the HiSeq 2500 (Illumina) with the Paired-End 100-bp protocol. The sequenced reads were trimmed off the adaptor sequences using Cutadapt (Matin, 2011). Trimmed reads with a length of 18–34 nt were mapped to the reference TAIR10 genome using Bowtie alignment algorithm (Langmead *et al*., 2009) without mismatch. The read data were submitted to the DDBJ Read Archive (Accession number DRA004828).

DNA methylation analysis

Genomic DNA was isolated from callus using the Nucleon PhytoPure (GE Healthcare Life Science). For DNA gel blot assays, 2.4 μg of DNA was digested with *Hpa*II, *Msp*I, or *Alu*I (New England Biolabs, Ipswich, MA, USA) and separated on 0.8% agarose gels. Hybridization was performed as described previously (Miura et al. 2004) using a specific probe (Table S8).

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Figure legends

Figure 1. Callus induction from different tissues of *Arabidopsis thaliana*. (a–c) Callus induction from hypocotyl. (a) A hypocotyl was separated from 7-d-old heat-stressed seedling and transferred to callus induction medium (CIM). (b) Callus was transferred to shoot induction medium (SIM) after 5 d of incubation on CIM. (c) Regenerated tissue after 16 d of incubation on SIM. (d–f) Callus induction from cotyledon. (d) A cotyledon was separated from 7-d-old heat-stressed seedling and transferred to CIM. (e) Callus was transferred to SIM after 7 d of incubation on CIM. (f) Regenerated tissue after 14 d of incubation on SIM. (g–i) Callus induction from seed. (g) Seeds were plated on CIM for 2 weeks, and the induced callus were subjected to heat stress. (h) Heat-stressed callus was transferred to SIM after 14 d of incubation on CIM. (i) Regenerated tissue after 2 d of incubation on SIM. (j) Graphic display of callus induction and heat stress treatment. Heat activation of *ONSEN* was induced at seedlings (a–f) and calli (g–i) independently.

519 **Figure 2.** Detection of new *ONSEN* copies in a callus-derived plant and the progeny. (a–c) Southern blot
520 of a wild-type plant regenerated from hypocotyl-derived callus (a), cotyledon-derived callus (b),
521 seed-derived callus (c) and its respective progenies. New-insertion specific bands were detected on callus
522 and the progenies subjected to heat stress. Same insertion patterns in (b) indicate the single origin of an
523 inserted cell. (d–f) Southern blot of a *nrpd1* plant regenerated from hypocotyl-derived callus (d),
524 cotyledon-derived callus (e), seed-derived callus (f) and its respective progenies. Control, wild-type plant;
525 NS, non-stressed; HS, heat stressed. A gel stained with ethidium bromide (EtBr) was used as a loading
526 control.
527
528 **Figure 3.** (a) Mapping of *ONSEN*-integrated genes in the wild-type progeny of a regenerated plant.
529 Twenty new copies were mapped within genes in *Arabidopsis thaliana*. Locus names in black font
530 indicate new insertions, whereas in red font the original *ONSEN* copies. (b) Graphic display of the
531 expression profiling data of *ONSEN*-inserted genes obtained from microarray analysis. A scatter plot of
532 the log2-transcript level values of the wild-type progeny versus those of the progeny of an
533 *ONSEN*-inserted line.
534
535 **Figure 4.** Relative transcript level of RdDM-related genes in seedlings and callus before the heat stress
536 (NS), immediately after the heat stress (HS), and two weeks after the heat stress (HS + 2 wk). Asterisks

mark significant differences compared with wild-type seedlings in each treatment ($P < 0.05$).

Figure 5. Changes in the abundance of small RNA in *ONSEN* before and after the heat stress. (a) Browser views of small RNA abundance around the *ONSEN* transposon (*AT1G13205*/ *AT5TE15240*) in wild-type seedlings and callus. The depth of mapped sequences is shown on the right side. (b) Changes in the abundance of small RNA before and after the heat stress in the *AT5TE15240* of seedlings and callus. Small RNA abundance is expressed in reads per million (RPM). RNA was collected before the heat stress (NS), immediately after the heat stress (HS), and two weeks after the heat stress (HS 2 wk).

Figure 6. Gene expression and DNA methylation in callus. (a) Relative transcript level of *HsfA2* in seedlings and callus before the heat stress (NS), immediately after the heat stress (HS), and two weeks after the heat stress (HS + 2 wk). (b) Relative transcript level of *ONSEN* in seedlings and callus before the heat stress (NS), immediately after the heat stress (HS), and two weeks after the heat stress (HS + 2 wk). Asterisks mark significant differences compared with wild-type seedlings ($P < 0.05$). (c) Methylation analysis of *ONSEN* by DNA gel blotting of NS and HS callus. Arrowheads indicate the extrachromosomal DNA of *ONSEN*. A gel stained with ethidium bromide (EtBr) was used as a loading control.

Figure 7. Callus induction and dedifferentiation in *Raphanus sativus*. (a) Seeds were plated on pre-culture

medium. (b) Seedling were plated on callus induction medium (CIM)1 after 9 d of incubation on pre-culture medium. (c) Seedlings were plated on CIM2 after 16 d of incubation on CIM1. (d) Dedifferentiated tissue induced by hypocotyl. (e) Dedifferentiated callus was isolated and plated on shoot induction medium (SIM) after 21 d of incubation on CIM2. (f) Shoot-induced callus was transplanted to soil after 14 d of incubation on SIM. (g) Regenerated *Raphanus sativus*. (h) The progeny of the regenerated plants was produced by geitonogamy. (i) Detection of new *ONSEN* copies in regenerated plants and their progenies. NS, non-stressed callus; HS, heat-stressed callus. Arrowheads indicate new *ONSEN* insertions. A gel stained with ethidium bromide (EtBr) was used as a loading control.

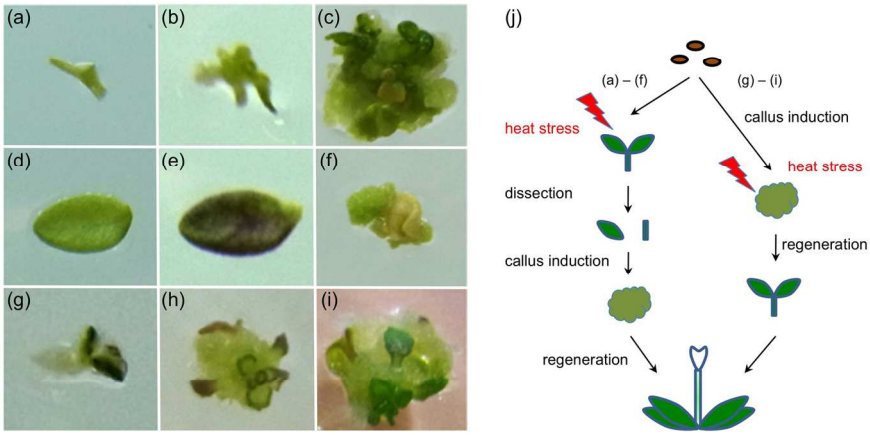

Fig.1

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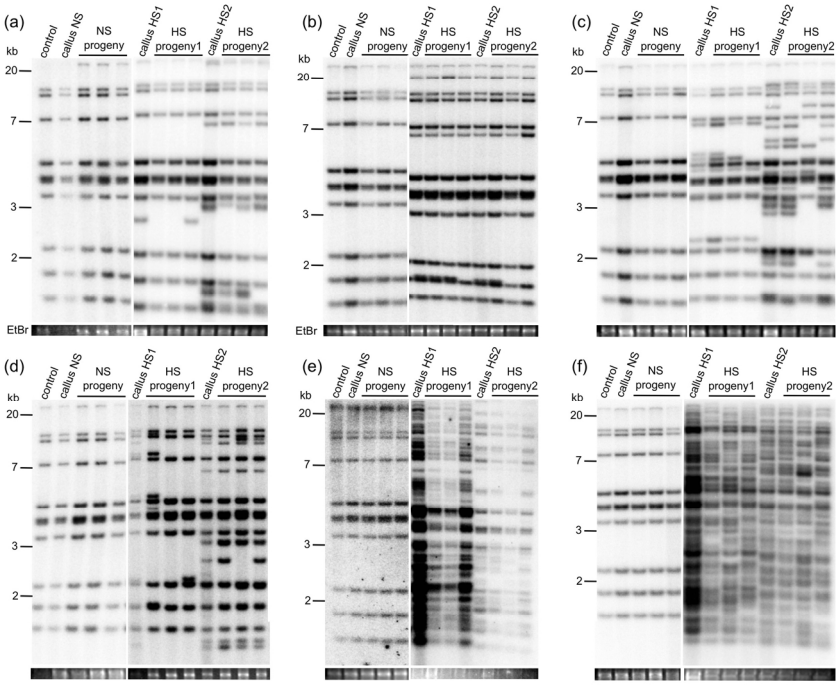

Fig.2

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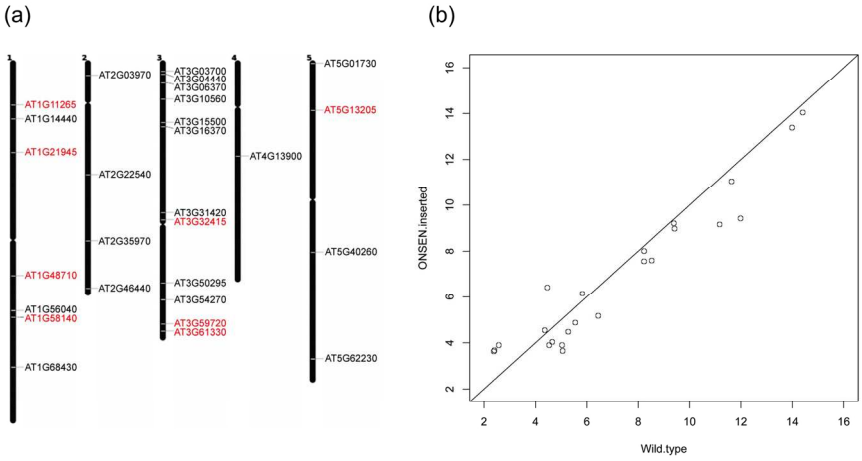

Fig.3

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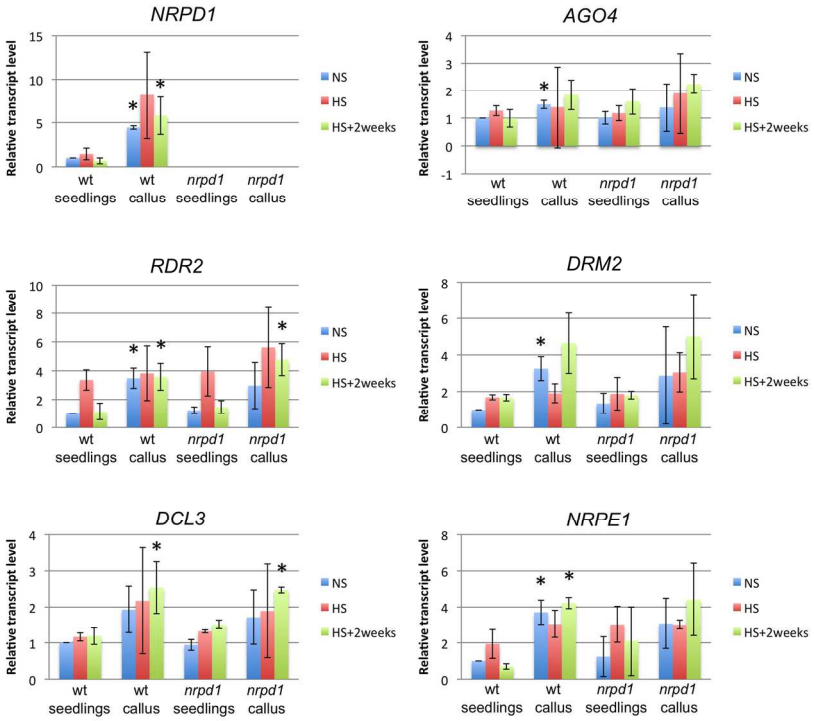

Fig.4

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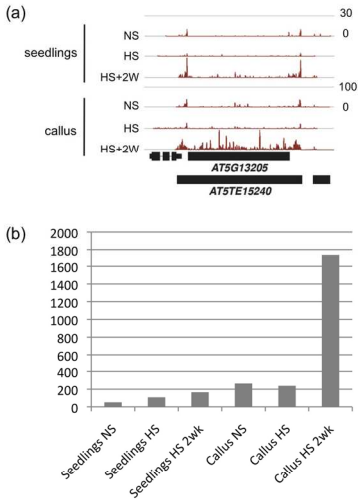

Fig.5

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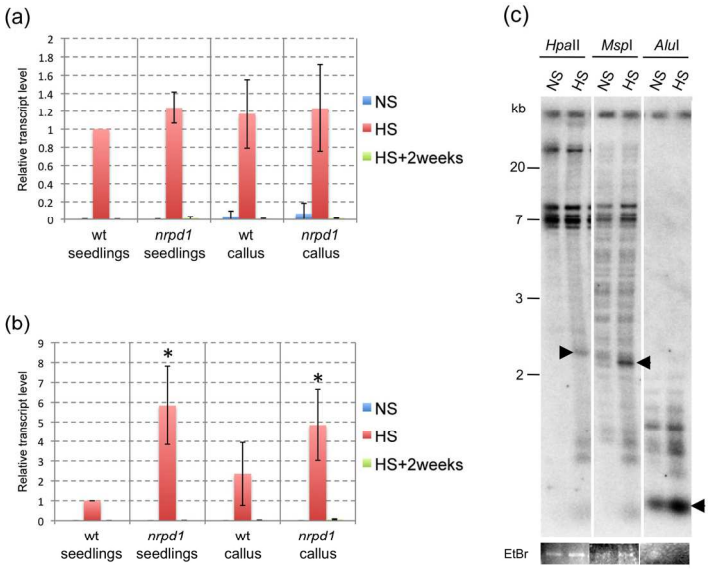

Fig.6

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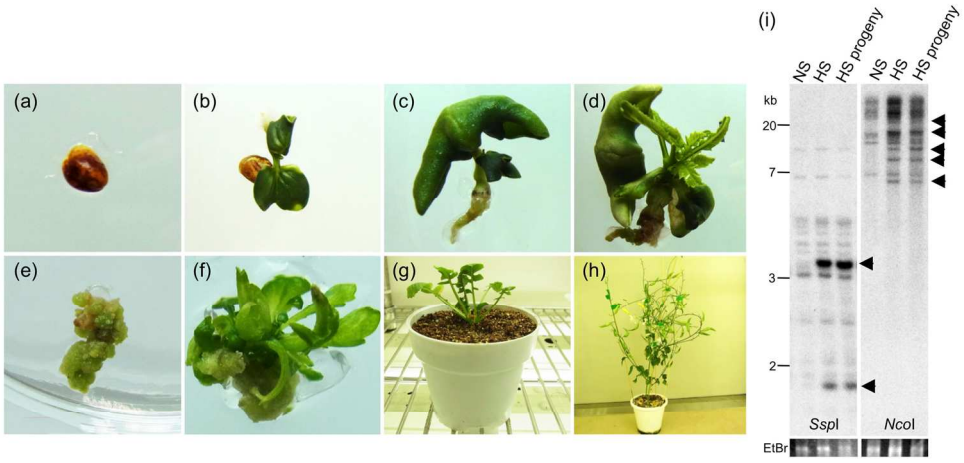

Fig.7

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