LETTER

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An siRNA pathway prevents transgenerational retrotransposition in plants subjected to stress

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Eukaryotic genomes consist to a significant extent of retrotransposons that are suppressed by host epigenetic mechanisms, preventing their uncontrolled propagation[1,2]. However, it is not clear how this is achieved. Here we show that in *Arabidopsis* seedlings subjected to heat stress, a *copia*-type retrotransposon named *ONSEN* (Japanese 'hot spring') not only became transcriptionally active but also synthesized extrachromosomal DNA copies. Heat-induced *ONSEN* accumulation was stimulated in mutants impaired in the biogenesis of small interfering RNAs (siRNAs); however, there was no evidence of transposition occurring in vegetative tissues. After stress, both *ONSEN* transcripts and extrachromosomal DNA gradually decayed and were no longer detected after 20–30 days. Surprisingly, a high frequency of new *ONSEN* insertions was observed in the progeny of stressed plants deficient in siRNAs. Insertion patterns revealed that this transgenerational retrotransposition occurred during flower development and before gametogenesis. Therefore in plants with compromised siRNA biogenesis, memory of stress was maintained throughout development, priming *ONSEN* to transpose during differentiation of generative organs. Retrotransposition was not observed in the progeny of wild-type plants subjected to stress or in non-stressed mutant controls, pointing to a crucial role of the siRNA pathway in restricting retrotransposition triggered by environmental stress. Finally, we found that natural and experimentally induced variants in *ONSEN* insertions confer heat responsiveness to nearby genes, and therefore mobility bursts may generate novel, stress-responsive regulatory gene networks.

In *Arabidopsis* mutants compromised in 24-nucleotide siRNA biogenesis, transposon transcripts appear but transposition has not been observed[3,4]. This is in contrast to mutants lacking chromatin-remodelling factor DDM1 or DNA methyltransferase MET1, in which transposons move during inbreeding[5–9]. It has been shown that transposon transcripts and their siRNAs accumulate in the vegetative nucleus of pollen[10]. A similar observation has been reported for the endosperm[11–13]. For pollen vegetative cells, where transposon mobility has been observed, it has been postulated that relocation of transposon siRNAs to sperm cells contributes to transposon silencing in the germ line[10]. Nevertheless, it is troubling that transposons remain immobile during inbreeding of mutants affected in siRNAs biogenesis, questioning the role of siRNAs in the control of germinal and, therefore, transgenerational transposon mobility[11].

We showed previously that a temperature shift applied to 1-week-old seedlings transiently destabilized transcriptional gene silencing (TGS) at loci residing within constitutive heterochromatin where TGS was re-established during the next 24 h[14]. A notable exception was a *Ty1/copia*-type long terminal repeat (LTR) retrotransposon family (*ATCOPIA78*), which retained high levels of transcripts two days later[14]. This was also observed in older plants (21 days) subjected to raised temperatures[15]. In the genome of the Columbia accession, *ATCOPIA78* consists of eight members (Supplementary Fig. 1a), hereafter referred to as *ONSEN*, of which three have identical LTR sequences, indicating recent transposition (Supplementary Fig. 1b).

By northern blotting, we compared *ONSEN* transcripts in seedlings subjected to a temperature shift of 24 h at 6 °C followed by 24 h at 37 °C (hereafter called heat stress (HS)) to transcripts of seedlings subjected to a control stress (CS) of 24 h at 6 °C followed by 24 h at 21 °C (Fig. 1a). *ONSEN* transcripts were detected in HS plants directly after the stress treatment and for up to 3 days of recovery at 21 °C (HS+3). The longest RNA found corresponded to the full-length transposon (Fig. 1a), whereas smaller RNAs appeared to belong to aberrant RNAs often associated with transcriptionally activated retroelements[9,16]. Full-length *ONSEN* transcripts were not observed in plants subjected to CS or in non-stressed plants (Fig. 1a and data not shown).

To examine further the specificity of TGS release and to determine possible epigenetic mechanisms involved in *ONSEN* control, we tested RNAs of plants treated with DNA methylation inhibitor 5-azacytidine (AzaC, Fig. 1b) and of *ddm1* mutant plants (Fig. 1c). Neither AzaC treatment nor *ddm1* mutation was effective for transcriptional activation of *ONSEN*, indicating that a reduction of DNA methylation is not sufficient for releasing *ONSEN* silencing. Furthermore, we applied HS and CS treatments to mutants compromised in epigenetic regulation (Fig. 1c, d). We examined *ddm1* mutants (Fig. 1c) and mutants affected in siRNA biogenesis (Fig. 1d): *nrpd1* (ref. 3), impaired in plant-specific RNA polymerase IV (PolIV); *nrpd2* (ref. 17), impaired in the common subunit of RNA PolIV and PolV; *rdr2* (ref. 18), impaired in RNA-dependent RNA polymerase 2; and *dcl3* (ref. 18), mutated in Dicer-like 3. We also challenged the *suvh2* (ref. 19) mutant (Fig. 1d), which is deficient in a putative histone 3 lysine 9 methyltransferase. *ONSEN* transcripts were only observed in RNA samples after HS but not after CS treatment (Fig. 1a, c, d). Their levels were not affected by the *ddm1* mutation (Fig. 1c). In contrast, HS-induced accumulation of *ONSEN* RNA was significantly higher in *nrpd1, nrpd2, rdr2, dcl3* and *suvh2* mutants (Fig. 1d). During the recovery period following stress, *ONSEN* transcripts diminished and after 10 days (HS+10) the full-length RNAs of the transposon were not detected on northern blots of all genotypes tested (Fig. 1a and Supplementary Fig. 2). These results indicated that siRNA-mediated regulation is responsible for the restriction of *ONSEN* transcript levels after HS, but is not involved in resilencing during the recovery period.

As PolIV is crucial for the biogenesis of the majority of 24-nucleotide siRNAs[4], we compared the levels of *ONSEN*-specific siRNAs in wild-type and *nrpd1* plants in relation to the HS-induced accumulation of its transcripts. Noticeably, directly after HS, when *ONSEN* transcript levels were highest, siRNA levels were low and increased only after 1 day of recovery (Fig. 1a, e, f). These siRNAs appeared in both wild-type and *nrpd1* plants and were mainly of the 21-nucleotide siRNA class (Fig. 1e, f), which is thought to direct cleavage of corresponding messenger RNAs. Although levels of 21-nucleotide siRNAs were significantly higher in *nrpd1* mutant plants than in the wild type, massive

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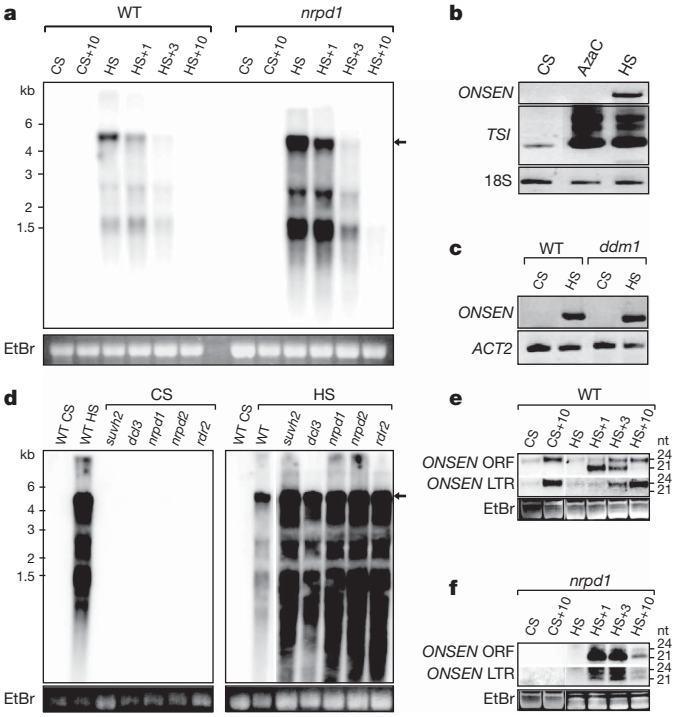

Figure 1 | Heat-stress induced *ONSEN* transcription. **a**, Northern blot revealing *ONSEN* transcripts in wild-type (WT) and *nrpd1* seedlings subjected to HS and after recovering from HS for 1, 3 and 10 days (HS+1, HS+3, HS+10); CS plants were subjected to the control stress (CS). The arrow marks the full-length *ONSEN* transcript. An ethidium bromide (EtBr)-stained gel is shown as loading control. **b**, Detection of *ONSEN* transcripts after AzaC and HS treatments by semiquantitative reverse transcription followed by PCR (RT–PCR) in wild-type plants. *TRANSCRIPTIONALLY SILENT INFORMATION* (*TSI*) was used as a positive control for the activation of heterochromatic transcription[14]; 18S ribosomal RNA was used as an internal control. **c**, Levels of *ONSEN* transcripts in *ddm1* mutant plants subjected to CS or HS and quantified by RT–PCR with *ACTIN2* transcripts (*ACT2*) as an internal control. **d**, Northern blots showing *ONSEN* transcript levels in selected mutants (marked above each lane) subjected to CS or HS. The CS blot was overexposed for possible detection of low levels of *ONSEN* transcripts. An EtBr-stained gel below is shown as a loading control. **e**, **f**, Northern blots of *ONSEN* siRNAs derived from open-reading frame (ORF) or LTR regions accumulating in wild-type (**e**) and *nrpd1* mutant plants (**f**). An EtBr-stained gel is shown as a loading control. nt, nucleotide. See Methods for probe information.

amounts of *ONSEN* transcripts were observed. Thus, this siRNA class was not able to prevent the accumulation of transposon-derived mRNA. However, the high background smear visible on northern blots (Fig. 1a, d) and even more apparent on overexposed blots of RNA isolated from mutants impaired in the siRNA pathway 10 days after HS (Supplementary Fig. 2) may be indicative of *ONSEN* transcript degradation. In the course of HS recovery (at 3 and 10 days), 24-nucleotide siRNAs highly accumulated in wild-type but not in *nrpd1* plants, and were especially abundant for the LTR regions of *ONSEN* (Fig. 1e, f). However, the contribution of 24-nucleotide siRNAs to resilencing at *ONSEN* loci during recovery is not clear, as they accumulate also in CS plants grown for an additional 10 days after CS treatment (Fig. 1e). Therefore their levels seem not to be related to the HS treatment. Moreover, *ONSEN* resilencing also occurred in *nrpd1,* where they were mostly absent (Fig. 1f).

Detection of *ONSEN* full-length transcripts, potentially able to serve as templates for reverse transcription, prompted us to examine the DNA of *Arabidopsis* subjected to HS. By Southern blot analysis we detected a significant increase in *ONSEN* copy number and observed a banding pattern indicative of the presence of two forms of extrachromosomal transposon copies, one linear reflecting the 2.8-kb fragment and one circular containing a single LTR consistent with the 4.5-kb fragment (Fig. 2a, left). Linear extrachromosomal forms are capable of chromosomal integration, in contrast to the circular forms that have been considered as by-products of retroelement replication[20,21]. In *nrpd1* and other mutant plants affected in the siRNA pathway, the abundance of HS-induced *ONSEN* DNA was significantly higher than in wild type (Fig. 2a, left). Noticeably, similarly high levels were observed in HS-treated *suvh2* mutant plants (Fig. 2a, left), which are known to exhibit wild-type levels of siRNAs[22]. After 10 days of recovery, *ONSEN* extrachromosomal DNA was still at a relatively high level but almost exclusively in the linear form (Fig. 2a, right).

Real-time quantitative polymerase chain reaction (qPCR) during HS and after subsequent recovery was performed to determine the kinetics of *ONSEN* DNA accumulation in wild-type plants and in a representative siRNA-biogenesis mutant (*nrpd1*) and to examine possible changes in copy number due to chromosomal integration events (Fig. 2b). Over the first 4 h of the temperature shift from 6 °C to 37 °C, the abundance of *ONSEN* DNA did not change. However, after 6 h, *ONSEN* copy number increased significantly from 8 endogenous copies to more than 30 in *nrpd1* but not in the wild type (Fig. 2b). After 12 h of HS, *ONSEN* copy number had increased in the wild type to more than 25 and in the *nrpd1* mutant to more than 160. The maximal copy numbers of over 50 for the wild type and over 500 for *nrpd1* mutants were reached 12 h and 24 h after HS, respectively. The HS-induced increase in *ONSEN* DNA seemed to be biphasic and this was especially pronounced in *nrpd1* plants (Fig. 2b). So far, we have no explanation for this biphasic accumulation but it may be related to stress-triggered synchronization of the retroelement replication cycle.

During 20–30 days of subsequent growth of both wild-type and *nrpd1* plants *ONSEN* copy number gradually decreased, reaching the initial number of the Columbia accession (Fig. 2b), consistent with the absence of or only sporadic chromosomal integration events. To examine whether new somatic integrations occurred, we performed transposon display on plants grown for 40 days after HS (Supplementary Fig. 3a). New *ONSEN* insertions were not detected in the genomic DNA of either wild-type or *nrpd1* mutant plants, consistent with the qPCR results. However, we can not exclude the possibility of rare transposition events occurring late in vegetative development leading to only small sectors with new insertions.

It has been suggested that transgenerational transposon mobility is suppressed during gametophyte formation by siRNAs[10,23]. However, there is no evidence based on germinal transposition events to support this hypothesis. By transposon display and Southern blot hybridization,

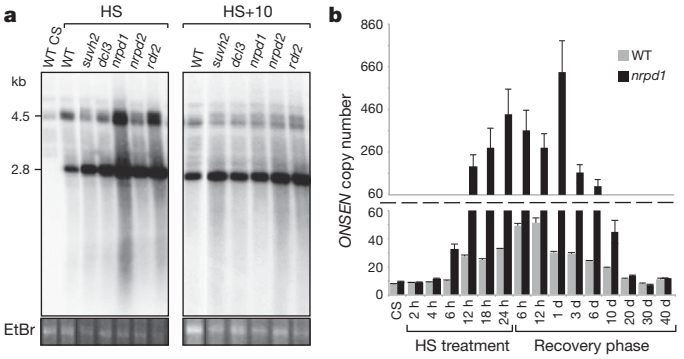

Figure 2 | Accumulation of *ONSEN* extrachromosomal DNA. **a**, Southern blot of PsiI-digested DNA isolated from HS-treated seedlings of wild type and selected mutants, directly after HS (left) or after 10 days of recovery (HS+10, right) and hybridized with an *ONSEN*-specific probe (see Methods). A 2.8-kb band is expected for the extrachromosomal linear form of *ONSEN* (Supplementary Fig. 1b). **b**, The kinetics of *ONSEN* DNA accumulation for wild type (grey) and *nrpd1* (black) measured by qPCR during and after HS treatment (mean ± s.e.m., $n = 3$ biological repetitions).

we examined genomic DNA from the progeny of self-fertilized wild-type and *nrpd1* plants subjected to HS and CS for new *ONSEN* insertions. Transposon movement was not detected in the offspring of either *nrpd1* or wild-type plants subjected to CS, or in HS-treated wild type (Fig. 3a and Supplementary Fig. 3b). However, a surprisingly high frequency of retrotransposition was recorded in the progeny of *nrpd1* mutant plants subjected to HS at the seedling stage (Fig. 3a and Supplementary Fig. 3b, c). Furthermore, the patterns of new *ONSEN* insertions in sibling seedlings derived from a single plant were found to differ in each individual examined, indicating that transposition occurred either before gametogenesis, during gametogenesis, after fertilization, or any combination therein (Fig. 3a and Supplementary Fig. 3b). To distinguish between these alternatives, we analysed *nrpd1* progeny plants derived from seeds of different flowers of the same progenitor (Fig. 3b). We found that patterns of new insertions differed entirely between progeny derived from different flowers. However, within the same flower we found common transposition patterns indicating somatic movement of *ONSEN* during flower development. Moreover, we were not able to find any new and unique *ONSEN* insertions specific to a single plant (Fig. 3b). Therefore all transposition events revealed in the sixteen progeny plants derived from two different flowers must have occurred before the differentiation of male and female gametophytes. Therefore, siRNA-mediated control of retrotransposon movement is not restricted to the gametophytic phase as it has been postulated[10–13].

To define better the roles of sporophytic and gametophytic 24-nucleotide siRNAs in suppressing stress-induced transgenerational retrotransposition, we examined heterozygote *nrpd1* plants subjected to HS treatment. In these plants the biogenesis of siRNAs is unaffected in the sporophyte, but is deficient in 50% of the male and female gametophytes. We compared the progeny of homozygous *nrpd1* mutant plants subjected to HS with homozygous *nrpd1* mutant

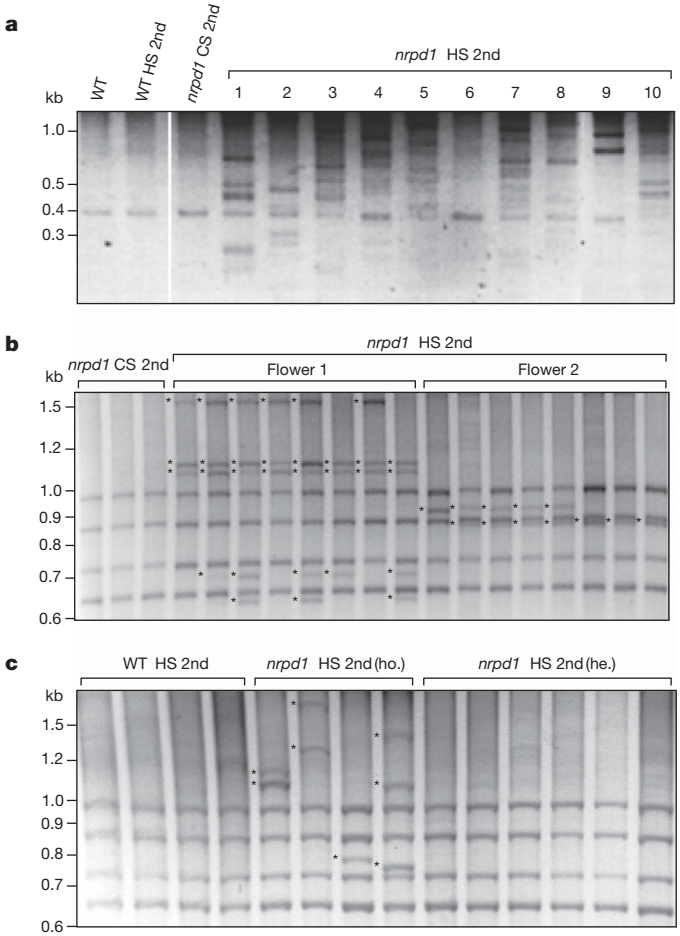

Figure 3 | Burst of *ONSEN* transposition in the progeny of HS-treated *nrpd1* plants. **a**, Transposon display (using primer Copia78 3′ LTR, Supplementary Table 2) detecting new *ONSEN* insertions. Numbers above the lanes of *nrpd1* HS 2nd (second generation) represent 10 individual plants that are siblings derived from bulk-harvested seeds of one *nrpd1* plant that was HS-treated as a 7-day-old seedling. **b**, Transposon display (using primer ONS_312_R, Supplementary Table 2) detecting new *ONSEN* insertions. Sixteen *nrpd1* HS 2nd plants are derived from two flowers of a single HS-treated *nrpd1* plant (flower 1 and flower 2 represented by eight plants each). Asterisks mark new *ONSEN* insertions. **c**, Transposon display (with primer as in **b**) revealing new *ONSEN* insertions in the progeny of HS-treated *nrpd1* homozygote mutant plants (*nrpd1* HS 2nd (ho.)) but not in the progeny of wild-type HS-treated plants (WT HS 2nd) or in *nrpd1* homozygote mutant progeny of HS-treated *nrpd1* heterozygote mutants (*nrpd1* HS 2nd (he.)).

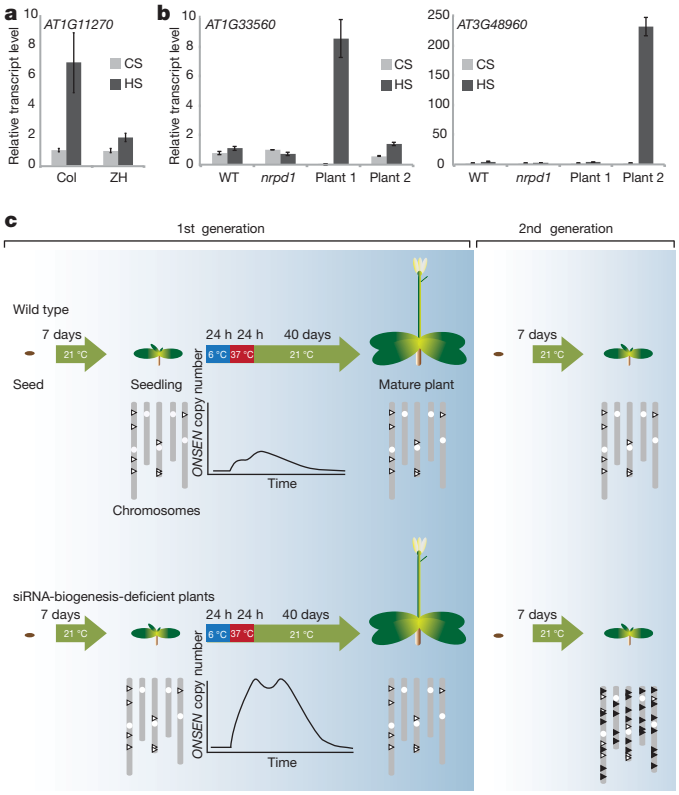

Figure 4 | Acquisition of heat-responsive transcription by *ONSEN*-containing loci and model of *ONSEN* activation. **a**, Effect of *ONSEN* natural insertion polymorphism on gene regulation. *AT1G11270* transcript levels after CS (grey) and HS (black) treatments were analysed by qRT–PCR in Columbia (Col) and Zürich (ZH) accessions in which *ONSEN* is present or absent at this locus, respectively (mean ± s.e.m., $n = 3$, values relative to *ACT2*). **b**, HS-mediated transcriptional regulation of two different genes acquiring new *ONSEN* insertion in the course of our experiments. Two progeny plants of HS-treated *nrpd1* homozygote mutant plant, named plant 1 and plant 2, were selected for displaying new homozygous *ONSEN* insertion at two distinct loci, *AT1G33560* and *AT3G48960*, respectively. Acquired transcriptional responses to HS of the affected genes were revealed by qRT–PCR (legend and values as in **a**). **c**, Summary of experimental results illustrating the role of the siRNA pathway in transgenerational control of *ONSEN* mobility. Upper part of the figure represents wild-type control of *ONSEN* activity and lower part illustrates uncontrolled accumulation of *ONSEN* copy number in siRNA-biogenesis-deficient plants. The graphs under the arrows illustrate the kinetics of *ONSEN* DNA accumulation on HS treatment. The open triangles on five *Arabidopsis* chromosomes represent eight endogenous *ONSEN* copies in the Columbia accession. The black triangles illustrate new *ONSEN* insertions found in the second generation. White circles on the chromosomes specify the location of the centromeres.

segregants derived from HS-treated *nrpd1* heterozygotes. A high frequency of retrotransposition was only observed in progeny of homozygous *nrpd1* mutant plants (Fig. 3c). These results are consistent with the involvement of 24-nucleotide siRNAs in either erasing 'stress memory' during somatic growth and/or suppressing retrotransposition in flower tissues, rather than with epigenetic control of retrotransposon movement during gametogenesis.

To define better the molecular mechanism controlling *ONSEN* transposition primed by HS, we analysed progenies of further HS-treated plants compromised in epigenetic regulation. Because DDM1 and KRYPTONITE (KYP, histone H3 lysine 9 methyltransferase) were previously implicated in transposition control of a related family of retrotransposons[6,9], we subjected both mutants to HS and examined the progeny by transposon display. Retrotransposition was not observed (Supplementary Fig. 4). Despite detecting high levels of transposon transcripts in *suvh2* mutants after HS (Fig. 1d) and a significant increase in *ONSEN* copy number (Fig. 2a), no retrotransposition events were found in the next generation (Supplementary Fig. 4). We next investigated mutants deficient in siRNA biogenesis (*nrpd2, rdr2* and *dcl3*). Transposition events were observed in *nrpd2* and *rdr2* (Supplementary Fig. 4), further indicating that biogenesis of siRNAs is crucial for preventing transgenerational mobility of *ONSEN*. As *dcl3* is essential for 24-nucleotide siRNA biogenesis, we predicted that there would be new *ONSEN* insertions in the progeny of HS-treated *dcl3* plants; in fact, no new insertions were detected (Supplementary Fig. 4). Therefore, although DCL3 clearly restricts the levels of *ONSEN* transcripts after HS, it is dispensable for the control of transgenerational transposition. This hints at two steps in *ONSEN* control: restraining levels of its transient transcription/reverse transcription and suppression of transgenerational transposition. As only the first requires DCL3 and SUVH2, the two control steps seem to be, at least in part, mechanistically independent. Given the functional redundancy of dicer-like (DCL) proteins in *Arabidopsis*[24], DCL3 may be substituted possibly by another DCL protein(s) at the second control step. It is also possible that transgenerational control of retrotransposition can occur without the involvement of dicer-like activities, as has been described in animals[25].

To determine whether *ONSEN* has preferential insertion targets, we characterized 11 new insertion sites and concluded that, although the retroelement inserted genome wide (Supplementary Fig. 5), it showed a clear preference for transcribed gene regions (all 11 insertions), with a further preference for exons (10 insertions) (Supplementary Table 1). Moreover, 2 of 11 insertions were homozygous (data not shown), which is consistent with retrotransposon movement during flower development but before the differentiation of anthers and carpels.

It has been postulated that a burst of transposition helped to shape plant genomes[26,27] and to modify their transcriptional responses[28]. Interestingly, a gene in the Columbia accession harbouring a natural insertion of *ONSEN* was identified as being heat responsive[29]. To determine the physiological relevance of this observation we analysed heat responsiveness of this gene in the Zürich accession where *ONSEN* is absent at this location (Fig. 4a). Indeed, HS-induced transcriptional activation in the Columbia accession was much more pronounced than in the Zürich accession (Fig. 4a). We determined also whether our experimentally induced retrotransposition events, in the second generation of *nrpd1* HS-treated plants, had an impact on the transcriptional regulation of endogenous loci harbouring new *ONSEN* insertions. We examined the heat-stress response of two such genes and showed that they became heat responsive when compared to wild-type or *nrpd1* first-generation plants (Fig. 4b). Therefore, it can be predicted that after our experimental burst of *ONSEN* transposition different subsets of genes in various progeny plants will acquire such regulatory properties. Now, having established an environmentally inducible system of transgenerational retrotransposition and having revealed the molecular and developmental mechanisms of its control (Fig. 4c), we are in a position to reproduce retrotransposon bursts in a controlled fashion and to determine their adaptive and/or damaging power.

METHODS SUMMARY

Plant material. All mutants used in this study (*dcl3-1* (ref. 18), *ddm1-2* (ref. 30), *nrpd1a-3* (ref. 3), *nrpd2a-2/2b-1* (ref. 17), *rdr2-2* SALK_059661 (ref. 18), *suvh2* SALK_079574 (ref. 19)) are in the Columbia (Col-0) background.

Stress treatment. Plants were grown in ½ MS medium (0.8% agar, 1% sucrose) in a Percival CU-22L chamber at 21 °C with 12 h light (140 μmol m^{-2} s^{-1}) and 12 h dark. After CS or HS treatment (see text), plants were grown at 21 °C in long-day conditions (16 h light). To analyse the progeny of CS- or HS-treated seedlings, plants were transplanted 10 days after CS/HS to soil and grown under long-day conditions.

RNA, DNA, and transposon display analyses. See Methods for details.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Plant material. All mutants used in this study (*dcl3-1* (ref. 18), *ddm1-2* (ref. 30), *nrpd1a-3* (ref. 3), *nrpd2a-2/2b-1* (ref. 17), *rdr2-2* SALK_059661 (ref. 18), *suvh2* SALK_079574 (ref. 19)) are in the Columbia (Col-0) background.

Stress treatment. Plants were grown in ½ MS medium (0.8% agar, 1% sucrose) in a Percival CU-22L chamber at 21 °C with 12 h light (140 μmol m^{-2} s^{-1}) and 12 h dark. After CS or HS treatment (see text), plants were grown at 21 °C in long-day conditions (16 h light). To analyse the progeny of CS- or HS-treated seedlings, plants were transplanted 10 days after CS/HS to soil and grown under long-day conditions.

RNA analysis. RNA was isolated from aerial parts of around 20 plants and northern blots, RT–PCR and siRNA analyses were carried out as described previously[9]. Full-length transcripts were detected with *ONSEN*-specific probe A, and siRNAs were detected with *ONSEN*-specific probe B (LTR region) or probe C (ORF). qRT–PCR analyses were performed using the Quantifast Multiplex PCR Kit (Qiagen). RNA levels were determined using TaqMan assays (qPCR thermocycler 7900HT, Applied Biosystems) and normalized using *ACTIN2*. PCR conditions were 95 °C for 5 min followed by 45 cycles alternating 45 s at 95 °C and 45 s at 60 °C. Probe localization and primer details are given in Supplementary Fig. 1b and Supplementary Table 2, respectively.

DNA analysis. Aerial parts from around 20 plants were collected and DNA was isolated with MiniPrep Kit (Qiagen) following the manufacturer's recommendations. Southern blots were performed as described previously[9] using *ONSEN*-specific probe (probe C, see Supplementary Fig. 1b and Supplementary Table 2). For qPCR analysis of *ONSEN* DNA copies, the Quantifast Multiplex PCR Kit (Qiagen) was used and *ACTIN2* was used to normalize DNA levels. DNA copy number was determined using TaqMan assays performed in the qPCR thermocycler 7900HT (Applied Biosystems) in a final volume of 10 μl. PCR conditions were 95 °C for 5 min followed by 45 cycles alternating 45 s at 95 °C and 45 s at 60 °C (primer details in Supplementary Table 2).

Transposon display. A simplified transposon display method based on the GenomeWalker Universal kit (ClontechLaboratories) was developed for library construction, with the following modifications. Genomic DNA (300 ng) was digested overnight with the blunt cutting DraI restriction enzyme (Promega) in a final volume of 50 μl, using a tenfold enzyme excess compared with the manufacturer's recommendations. After digestion, DNA fragments were purified on a PCR purification column (Qiagen) following the manufacturer's instructions and eluted into 20 μl; 5 μl was used for overnight ligation at 16 °C in 16 μl with GenomeWalker adaptors. After ligation, DNA was diluted 20-fold and 1 μl used as a template for the PCR reaction. PCR were performed using a primer specific for *ONSEN* (Copia78 3′ LTR or ONS_312_R) and a primer specific for the adaptor (GenWalk_AP1). PCR conditions were 5 min at 95 °C, followed by 33 cycles of 30 s at 94 °C, 30 s at 58 °C, 1 min at 72 °C; and a final elongation step of 7 min at 72 °C. PCR products were separated on 3% agarose gels. Primer details are given in Supplementary Table 2.