

The effect of zebularine on the heat-activated retrotransposon *ONSEN* in *Arabidopsis thaliana* and *Vigna angularis*

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The Ty1/copia-like retrotransposon *ONSEN* is conserved among *Brassica* species, as well as in beans, including adzuki bean (*Vigna angularis* (Willd.) Ohwi & Ohashi), which is one of the economically important crops in Japan. *ONSEN* has acquired a heat-responsive element that is recognized by plant heat stress defense factors, resulting in its transcription and the production of full-length extrachromosomal DNA under conditions with elevated temperatures. DNA methylation plays an important role in regulating the activation of this transposon in plants. Therefore, chemical inhibition of DNA methyltransferases has been utilized to study the effect of DNA methylation on transposon activation. To understand the effect of DNA methylation on *ONSEN* activation, *Arabidopsis thaliana* and adzuki bean seedlings were treated with zebularine, which is known to be an effective chemical demethylation agent. The results showed that *ONSEN* transcription levels were upregulated in zebularine-treated plants. Extrachromosomal DNA of *ONSEN* also accumulated in the treated plants.

Key words: adzuki bean, *Arabidopsis*, heat stress, *ONSEN*, zebularine

INTRODUCTION

Transposable elements (TEs) are major components of the genomes of many eukaryote species (Wessler, 1996). TEs are divided into two classes: class I TEs include retrotransposons, which generally function via reverse transcription by a copy-and-paste mechanism; and class II TEs include DNA transposons, which encode the protein transposase that is required for their insertion and excision via a cut-and-paste mechanism. The present study focused on class I TEs (retrotransposons), which are divided into two orders based on the presence or absence of a long terminal repeat (LTR) at either end of the element (Finnegan, 1989). DNA copies of LTR retrotransposons are inserted by an integrase enzyme that is encoded by the element itself. During this process, staggered cuts are first made at the target site. Extra nucleotides from the 3' termini of the LTRs are then trimmed, and finally the 3' termini are joined to the free 5' ends at the site of the staggered cut (Schulman, 2012).

The lifecycle of an LTR retrotransposon begins when

retrotransposon RNA is reverse transcribed in the nucleus. The transcripts are exported to the cytoplasm, and are then either translated into the polypeptide encoded by the retrotransposon or reverse transcribed again. The RNA template is packaged into virus-like particles (VLPs) consisting of *gag* gene products associated with transcripts of the LTR retrotransposon and the *pol* gene that encodes integrase, reverse transcriptase and RNase H domains. The *gag* and *pol* genes overlap, meaning that translation of *pol* RNA requires a frameshift, as is the case for retroviral transcripts. A VLP containing RNA is reverse transcribed to form cDNA, and then the resultant VLP containing cDNA is imported into the nucleus, after which integration takes place (Saariaho, 2006). LTR retrotransposons are divided into two main superfamilies, *gypsy* and *copia*, which most likely originated from two independent gene fusion events.

In flowering plants, the major small RNA-mediated epigenetic pathway is RNA-directed DNA methylation (RdDM). RdDM includes two specific DNA-dependent RNA polymerases, namely Pol IV, which produces an initial RNA transcript for RNA silencing, and Pol V, which transcribes a messenger RNA that contributes to the small interfering RNA (siRNA)-induced DNA methylation of the target site. RNA-dependent RNA poly-

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merase 2 transcribes TEs into double-stranded RNA, which the enzyme Dicer-like 3 then processes to form 24–26-nucleotide siRNAs. The siRNAs bind to the Argonaute 4 (AGO4) gene that interacts with Pol IV to recruit DNA methyltransferase (DRM2), which plays an important role in the management of enzymatic processes in the RdDM pathway that lead to *de novo* DNA methylation of target TEs (Matzke and Mosher, 2014). DNA methylation is meiotically inherited, and can thus affect morphological variation among the offspring of an organism (Ji et al., 2015).

One of the Ty1/*copia*-like retrotransposons, named *ONSEN*, is activated by heat stress in *Arabidopsis thaliana*. This occurs because *ONSEN* contains a heat-responsive element that is recognized by plant-derived heat stress defense factors, and results in transcription and the production of full-length extrachromosomal DNA (ecDNA) under conditions with elevated temperatures (Ito et al., 2011).

Ty1/*copia*-like retrotransposons have been found in a wide variety of legume crops. For example, an LTR retrotransposon named *Panzee* has been reported to occur in pigeon pea (*Cajanus cajan* (L.) Millsp.) (Lall et al., 2002). In addition, two Ty1/*copia*-like retrotransposon families, *pvRetro3* and *pvRetro4*, were found to have an extra open reading frame (ORF) located between the retrotransposase ORF and the 3' LTR in common bean (*Phaseolus vulgaris* L.) (Gao et al., 2014). These LTR retrotransposons also encoded an envelope-like protein, which has rarely been identified in such elements in plant genomes (Gao et al., 2014). Another example of a Ty1/*copia*-like retrotransposon is *SORE-1*, which has been found in soybean (Liu et al., 2008). This element affects the transcription of the gene that encodes phytochrome A, and confers photoperiod sensitivity (Liu et al., 2008). Adzuki bean (*Vigna angularis* (Willd.) Ohwi & Ohashi) is one of the economically important crops in Japan, especially in the Hokkaido region. Mutagenesis using TEs may be a useful technique to enhance the genetic diversity of this bean crop. Since the full-length genome sequence of adzuki bean is available, this provides a good opportunity to search for transposable elements in this plant (Sakai et al., 2015). Moreover, it has been reported that the heat-activated *ONSEN* element is expressed in two varieties of adzuki bean, 'Shumari' and 'Tanba-dainagon' (Masuta et al., 2018). However, ecDNA of this element has been detected only in the 'Tanba-dainagon' variety.

Chemical inhibition of DNA methyltransferases has been utilized to study the effects of DNA methylation in plants. The chemical demethylating agents that have been used for this include 5-azacytidine and zebularine, which are both non-methylable cytosine analogs (Pecinka and Liu, 2014). These agents are incorporated into the DNA double helix in place of cytosine with each cycle of

DNA replication. Both agents inhibit DNA methylation in a similar manner, but zebularine has been shown to be more stable than 5-azacytidine. It has also been previously determined that zebularine is a more effective demethylating agent than 5-azacytidine (Baubec et al., 2009), which could be attributed to the weak binding between zebularine and DNA methyltransferases (Champion et al., 2010) or to its consolidating less frequently with the DNA double helix (Jones and Taylor, 1980).

Zebularine inhibits DNA methylation and increases the gene expression level of the transposon. For example, a correlation between *ONSEN* expression level and DNA methylation has been reported in *A. thaliana*. The results indicated that DNA methylation contributes to the repression of *ONSEN* transcript level and ecDNA production (Thieme et al., 2017). However, there is no report for adzuki bean and our work is the first study of the effect of zebularine on *ONSEN* expression, transposition and DNA methylation level in this species. We used wild type adzuki for studying the general effects of zebularine at different concentrations to find the optimal concentration, because each species has its own concentration that leads to maximal ecDNA production.

MATERIALS AND METHODS

Plant materials, growth conditions and heat stress treatment Wild-type *A. thaliana* seeds of the ecotype 'Columbia' (Col-0) and seeds of the adzuki bean (*V. angularis*) variety 'Tanba-dainagon' were obtained and sterilized by an ethanol-based method. Adzuki bean was grown on Murashige and Skoog (MS) medium plates. One-week-old seedlings were subjected to a temperature shift from 4 °C for 24 h to 40 °C for 24 h (*V. angularis*). After the heat treatment, DNA and RNA samples were extracted immediately from the seedlings. As a control, some seedlings were also subjected to a temperature shift from 4 °C for 24 h to 28 °C for 24 h (*V. angularis*).

Callus induction *Arabidopsis thaliana* seeds were plated on callus induction medium (CIM), containing 3.1 g/l of Gamborg's B5 salts (Sigma-Aldrich) with 0.5 g/l of 2-morpholinoethanesulfonic acid (MES), 20 g/l of glucose, 2.5 g/l of gellan gum, 0.05 mg/l of kinetin and 0.5 mg/l of 2,4-D, for two weeks. The induced calli were then subjected to a temperature shift from 21 to 37 °C for 24 h. The heat-stressed calli were transferred to shoot induction medium, containing 3.1 g/l of Gamborg's B5 salts with 0.5 g/l of MES, 20 g/l of glucose, 2.5 g/l of gellan gum, 5 mg/l of 6-(γ,γ -dimethylallylamino) purine and 0.1 mg/l of indole-3-acetic acid, and were then incubated for two weeks. The induced shoots were then transferred to root induction medium, containing 4.3 g/l of MS

medium with 0.5 g/l of MES, 10 g/l of sucrose, 2.5 g/l of gellan gum, 2 mg/l of 1-naphthaleneacetic acid and 1 ml/l of 1,000× B5 for 160 days and then the plant samples were collected.

Vigna angularis seedlings were grown on MS medium plates with continuous lighting at 27 °C for seven days. Subsequently, the temperature shift they experienced was from 4 °C for 24 h to 40 °C for 24 h. The hypocotyl of each heat-stressed plant was then transferred to CIM, including 4.4 g/l of MS with 0.5 g/l of MES, 30 g/l of sucrose, 0.1 mg/l of benzyladenine and 8 g/l of agar.

Chemical preparation Zebularine (Tokyo Chemical Industry, Japan) was dissolved in water before being added to liquefied, cooling agar at final concentrations of 0.01 and 0.04 mM (in plates containing *A. thaliana*) or 0.1, 0.3, 0.5 and 1.0 mM (in plates containing *V. angularis*).

Quantitative polymerase chain reaction (qPCR) Primers were designed to determine the copy number of *ONSEN* in Arabidopsis and adzuki bean (Supplementary Table S1). The 18S ribosomal RNA gene was selected as the reference gene and the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to perform qPCR. Three biological replicates were analyzed for each sample.

RNA extraction Total RNA was extracted from whole seedlings using TRI Reagent (Sigma Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. Approximately 3–5 µg of the total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). Reverse transcription was performed using the ReverTraAce qRT-PCR Kit (Toyobo, Osaka, Japan), with random primers.

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 previously (Miura et al., 2004) using an [α -³²P]dCTP-radiolabeled *ONSEN*-specific probe (Supplementary Table S1) generated using the Megaprime DNA Labeling System (GE Healthcare Life Science) in high sodium dodecyl sulfate hybridization buffer (Church and Gilbert, 1984). The DNA was digested with EcoRV, and then the resultant hybridization signals were detected.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) *ONSEN*-specific primers for adzuki bean were designed using Primer3Plus software (<https://primer3plus.com>) based on candidate gene coding sequence information (Supplementary Table S1). The 18S ribosomal RNA gene was selected and used as the reference gene. Luna Universal qPCR Master

Mix (New England Biolabs, Ipswich, MA, USA) and the Applied Biosystems 7300 Real-Time PCR System were used to perform the qRT-PCR. Three biological replicates were analyzed for each sample.

RESULTS

Detection of new *ONSEN* insertions in *A. thaliana* callus treated with zebularine and subjected to heat stress To detect new insertions of *ONSEN* that occurred after plants were treated with zebularine and subjected to heat stress, we conducted a Southern blot analysis using DNA extracted from the plants that were derived from callus of *A. thaliana* 160 days after heat stress. Many new bands were detected in the heat-stressed plants treated with 0.01 and 0.04 mM zebularine (Fig. 1). The relative copy number of *ONSEN* was calculated by qPCR to confirm the Southern results. The copy number of *ONSEN* in seedlings treated with 0.01 mM zebularine was 4.27-fold higher than that in the non-zebularine-treated seedlings subjected to heat stress, whereas there was no significant difference between the copy numbers in samples treated with 0.01 and 0.04 mM zebularine subjected to heat stress (Table 1). The results indicated that 0.01 mM zebularine could enhance the activation of *ONSEN* in *A. thaliana* callus.

The effect of zebularine on *ONSEN* expression in adzuki bean To analyze the effect of zebularine on *ONSEN* expression, we treated adzuki bean seedlings with various concentrations of zebularine (0, 0.1, 0.3, 0.5 and 1.0 mM). In medium containing 0.5 mM zebularine, the growth of one-week-old 'Tanba-dainagon' seedlings was obviously different between those treated with zebularine and non-treated seedlings. The treated seedlings showed dwarfism in terms of impaired stem and root elongation in a concentration-dependent manner. In addition, few lateral roots were detected in 0.1 mM zebularine-treated plants and only a small root was detected in plants treated with 0.3, 0.5 and 1.0 mM zebularine (Fig. 2). The sizes of the seedlings and root lengths were significantly lower in the zebularine-treated plants than in the non-treated plants (Table 2).

The transcript level of *ONSEN* was determined by qRT-PCR. The results showed that *ONSEN* expression was not activated without heat stress. The transcript level increased as the concentration of zebularine in the medium rose from 0 mM to 0.5 mM, but then declined in the treatment with 1.0 mM zebularine (Table 3). These results indicated that a concentration of 0.5 mM zebularine was capable of strongly activating *ONSEN* expression in 'Tanba-dainagon'.

To test the effect of zebularine on the methylation level of *ONSEN*, we performed Southern blotting using a methylation-sensitive restriction enzyme. The result showed

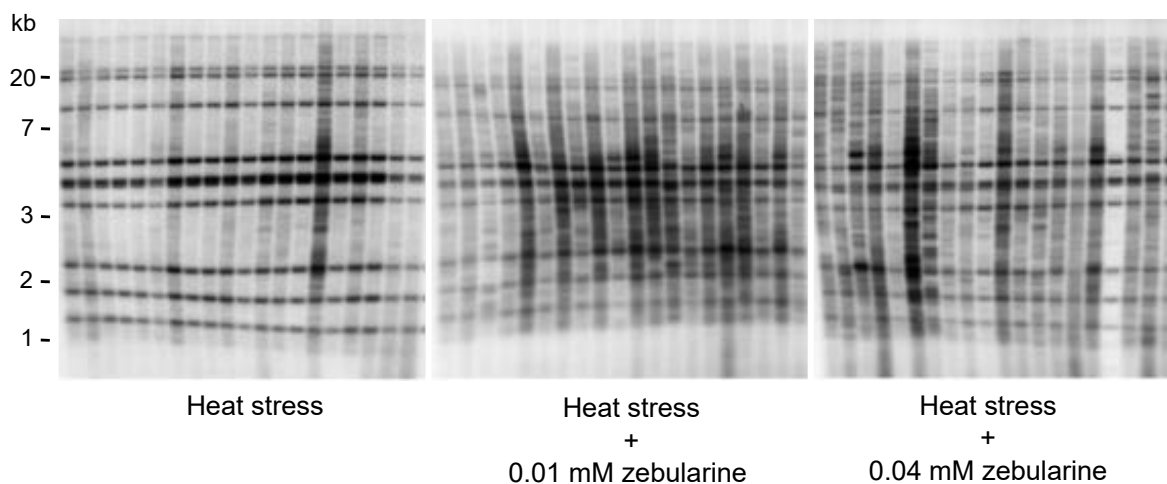


Fig. 1. Detection of new *ONSEN* copies in tissue that was derived from callus. The DNA was digested with EcoRV to detect new insertion of *ONSEN* in *A. thaliana*. The DNA was extracted from tissue that was derived from callus 160 days after heat stress under different concentrations of zebularine.

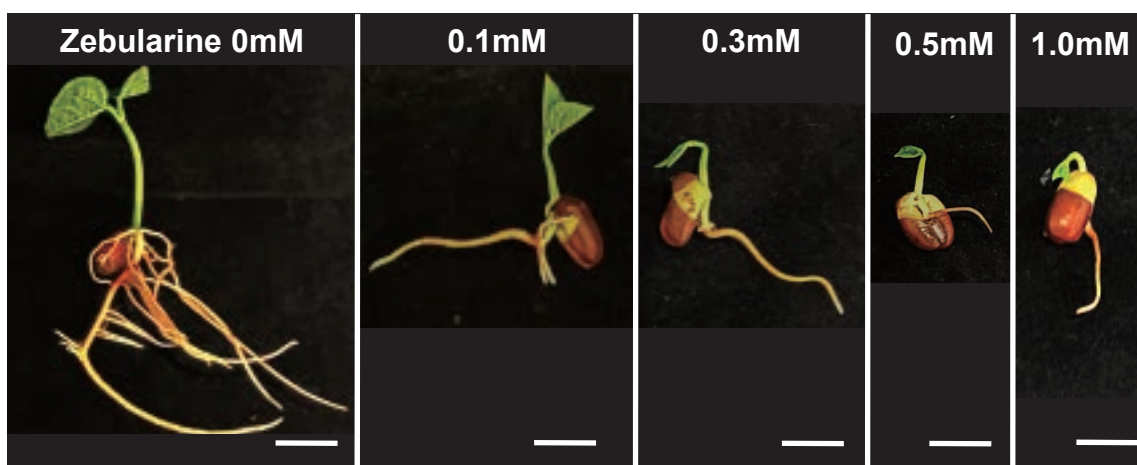


Fig. 2. Phenotype of one-week-old seedlings of adzuki bean exposed to zebularine. Scale bars, 1 cm.

Table 1. Relative copy number of *ONSEN* in *A. thaliana* callus

	Non-stress	Heat stress	Heat stress + 0.01 mM zebularine	Heat stress + 0.04 mM zebularine
Relative copy number	1.00 ± 0.09	1.86 ± 0.41	7.95 ± 0.46*	8.13 ± 2.78*

Data are presented as mean ± standard deviation (n = 3).

Asterisks mark significant differences from the copy number of *ONSEN* in non-stress samples ($P \leq 0.05$, ordinary one-way ANOVA).

Table 2. Size of one-week-old adzuki bean seedlings

	0 mM zebularine	0.1 mM zebularine	0.3 mM zebularine	0.5 mM zebularine	1.0 mM zebularine
Above-ground part	2.93 ± 0.15	1.83 ± 0.12*	1.67 ± 0.21*	1.20 ± 0.28*	1.17 ± 0.21*
Root	7.33 ± 0.40	3.80 ± 0.10*	3.80 ± 0.61*	1.65 ± 0.07*	2.17 ± 0.78*

Data are presented (in cm) as mean ± standard deviation (n = 3).

Asterisks mark significant differences from the size for 0 mM zebularine ($P \leq 0.05$, ordinary one-way ANOVA).

Table 3. Relative transcript level of *ONSEN* in adzuki bean seedlings

	0 mM	0.1 mM	0.3 mM	0.5 mM	1.0 mM
Heat-stressed	1.00 ± 0.39	15.35 ± 1.19	46.63 ± 9.69*	68.37 ± 1.90*	47.05 ± 23.05*

Data are presented as mean ± standard deviation (n = 3).

Asterisks mark significant differences from the transcript level of *ONSEN* in 0 mM zebularine, heat-stressed ($P \leq 0.05$, ordinary one-way ANOVA).

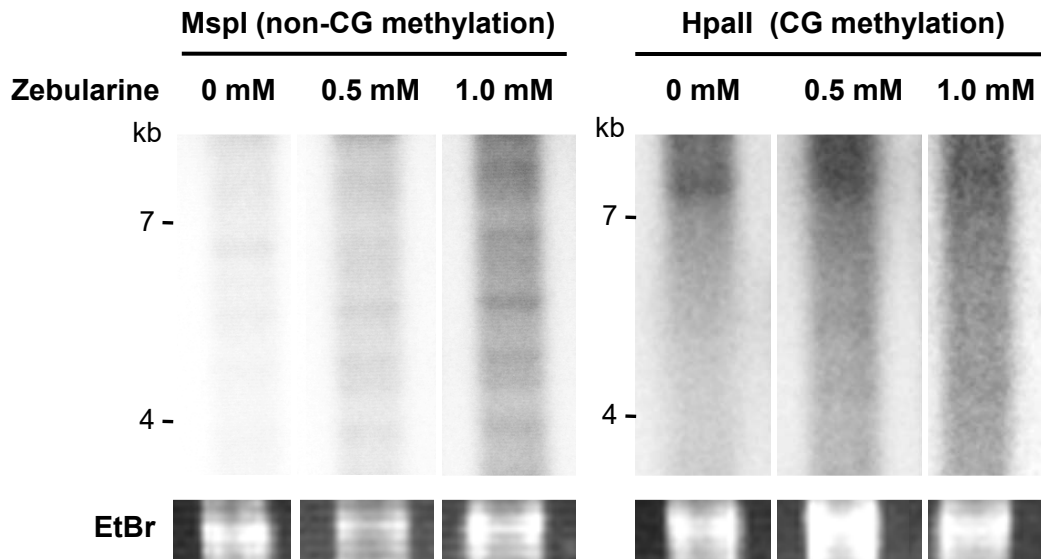


Fig. 3. Methylation analysis of *ONSEN* of adzuki bean. Seedlings exposed to zebularine were analyzed by Southern blotting after digestion of genomic DNA with a methylation-sensitive restriction enzyme. *HpaII* and *MspI* recognize the same restriction site (CCGG) but have different sensitivities to cytosine methylation. *HpaII* cannot cleave DNA if the cognate restriction sites are methylated, while *MspI* can cleave every restriction site regardless of its methylation and is included here as a control. The probe used for these Southern blots is the same as that used in Fig. 1. Smear bands with smaller size indicated hypomethylated *ONSEN* sequences. A gel stained with ethidium bromide (EtBr) was used as a loading control.

that the DNA methylation of *ONSEN* was reduced in the zebularine-treated adzuki (Fig. 3).

The effect of zebularine on *ONSEN* transposition in adzuki bean To test the transposition activity of *ONSEN* in adzuki bean plants treated with zebularine, we analyzed *ONSEN* ecDNA by Southern blot analysis. The ecDNA was detected in ‘Tanba-dainagon’ seedlings subjected to heat stress, and strong signals were detected in those treated with 0.5 mM zebularine (Fig. 4). This result was consistent with our analysis of *ONSEN* transcript levels in seedlings treated with 0.5 mM zebularine.

Detection of *ONSEN* copies in adzuki bean callus treated with zebularine and subjected to heat stress To compare the copy number of *ONSEN* in adzuki bean treated with or without zebularine and subjected to heat stress, we analyzed the copy number of *ONSEN* by qPCR using DNA extracted from the callus of ‘Tanba-dainagon’ 45 days after heat stress. The result

showed that the copy number of *ONSEN* in calli treated with 0.5 mM zebularine was 2.4 times higher than that in the non-zebularine-treated callus subjected to heat stress (Table 4). This result indicated that 0.5 mM zebularine could enhance the activation of *ONSEN* in adzuki bean callus.

DISCUSSION

When zebularine was used to inhibit DNA methylation in callus of *A. thaliana*, it was found that a concentration of 0.01 mM could increase the copy number of *ONSEN*. However, 0.04 mM zebularine did not induce higher accumulation of *ONSEN* than that seen in the 0.01 mM treatment. Regions enriched for CHH (where H is A, C or T) such as *ONSEN* are found to be completely demethylated in CHH contexts by treatment with 0.1 mM zebularine in *A. thaliana* (Griffin et al., 2016). Griffin et al.’s results also demonstrated that CG context methylation in 0.025 and 0.05 mM zebularine treatment was not significantly different, while 0.1 mM treatment

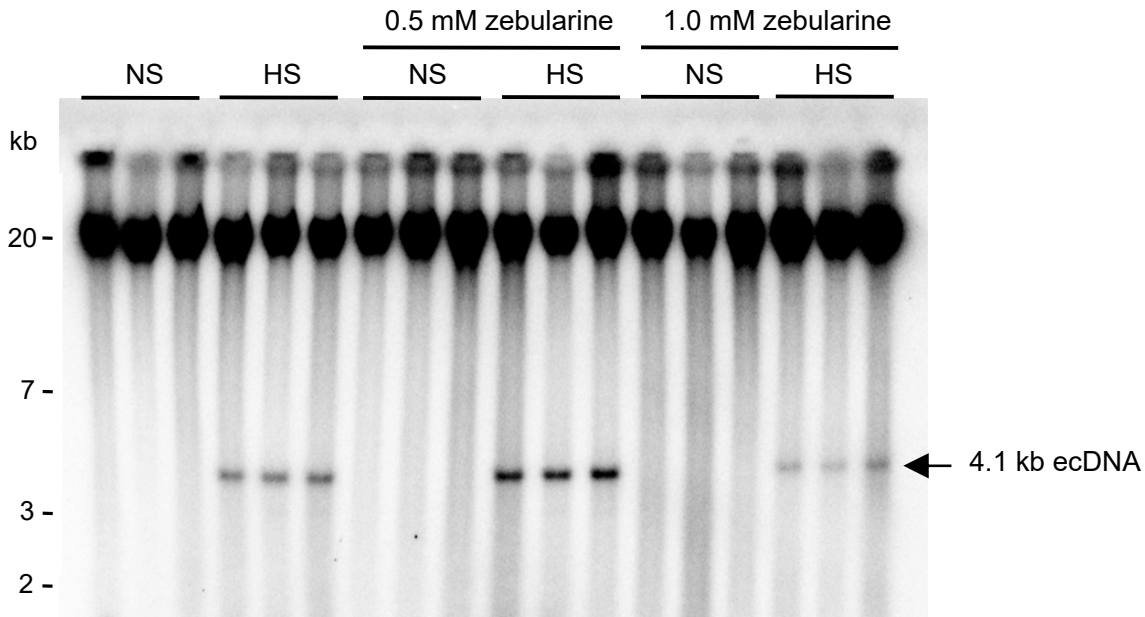


Fig. 4. Effect of zebularine on *ONSEN* activation in adzuki bean. Non-digested DNA extracted from the seedlings treated with 0 mM, 0.5 mM and 1.0 mM zebularine was loaded to detect ecDNA by Southern blotting. NS: non-stress, HS: heat-stressed plant. Arrow indicates ecDNA of *ONSEN*.

Table 4. Relative copy number of *ONSEN* in adzuki bean callus subjected to heat stress

	Non-treated	0.5 mM zebularine
Relative copy number	1.00 ± 0.09	2.43 ± 0.54

Data are presented as mean ± standard deviation (n = 3).

showed much decreased DNA methylation. Comparing this with our result that there was no significant difference between 0.01 and 0.04 mM zebularine treatment, it seemed likely that when the concentration exceeds 0.04 mM, the amount of extrachromosomal DNA increases, according to the inverse proportionality of DNA methylation and transcript level of *ONSEN*. Since *ONSEN* is enriched for CHH contexts, the *ONSEN* expression level, the ecDNA copy number and the DNA methylation level probably depend on CHH contexts. For DNA methylation results, the difference between Griffin et al.'s and our results may be attributable to the method used to grow the seeds: Griffin et al. applied a two-day seed stratification period at 4 °C before moving the seeds to room temperature and growing them for eight days, while we sowed the seeds and waited for seven days without seed stratification.

A previous study detected somatic transposition of *ONSEN* in calli of *Arabidopsis* and *Brassica* species after they were subjected to heat stress at 37 °C for 24 h (Ito et al., 2013). One of the benefits of callus-mediated transposition of *ONSEN* was that new alleles were generated, which thereby could help to expand the genetic variation of the tested plants. However, the presence of

somaclonal variation was also detected, and this method requires relatively costly manipulations (Masuta et al., 2017). In a previous study, Thieme et al. (2017) used seedlings instead of calli and measured *ONSEN* transcript level, ecDNA copy number and percent methylated cytosines after 0.04 mM zebularine treatment, which was claimed to be the most suitable concentration in *A. thaliana*. According to Masuta et al. (2018), the optimal heat stress condition for adzuki bean was 40 °C for 24 h. ecDNA was not detected in seedlings subjected to 37 °C for 24 h, which indicated that the optimal temperature for activating *ONSEN* varies among species. The appropriate concentration of zebularine to activate *ONSEN* was also different among species. The *ONSEN* expression level in adzuki bean does not always depend on the concentration of zebularine, which correlates with a study in *A. thaliana* and *Medicago sativa* (Baubec et al., 2009). In the present study, the highest concentration tested did not result in the highest level of *ONSEN* expression: treatment with 0.5 mM zebularine resulted in the highest *ONSEN* expression level, while the expression level declined from this peak in the treatment with 1.0 mM zebularine. Zebularine was previously found to be more stable in aqueous solution and less cytotoxic than 5-azacytidine (Cheng et al., 2004). Zebularine has also been reported to inhibit DNA methyltransferases (e.g., DRM2), leading to decreased *ONSEN* DNA methylation and subsequent activation of the transcription of this element, resulting in increased RNA polymerase II (Pol II)-dependent production of the sense and antisense transcripts thereof (Thieme and Bucher, 2018).

According to Masuta et al. (2018), *ONSEN* is expressed in two varieties of adzuki bean, ‘Tanba-dainagon’ and ‘Shumari’. However, ecDNA of *ONSEN* was only detected in the ‘Tanba-dainagon’ variety (Masuta et al., 2018). Consequently, based on previous studies, we applied different concentrations of zebularine to adzuki bean plants of the latter variety subjected to heat stress. However, the ‘Tanba-dainagon’ variety had its own optimal zebularine concentration level for *ONSEN* expression, and the expression level decreased when a concentration exceeding this optimum one was applied. The detected full-length ecDNA was produced by assembly into VLPs. If there were no frame-shift mutations or stop codons in the coding sequence regions, this ecDNA would be able to integrate into a new locus or recombine with pre-existing elements (Servant and Deininger, 2016). The strength of the signal detected during Southern blotting was also highest in the 0.5 mM treatment, and lower in that with 1.0 mM of zebularine, which agreed with the results for *ONSEN* expression levels. DNA methylation on transposons is controlled by the RdDM pathway. The DNA hypomethylation in non-CG contexts was concentration-dependent. Although the *ONSEN* promoter contains only CHH sites, all sequence contexts are typically methylated (Cavrak et al., 2014). According to Griffin et al. (2016) and Thieme et al. (2017), the methylated cytosine was decreased after zebularine treatment. In addition, maintenance of cytosine methylation at CHH sites in RdDM is necessary for dsRNA stimulation (Vaucheret, 2006). Therefore, our results confirmed that the effect of zebularine correlated with the RdDM pathway. CG, CHG and CHH methylation status in each species or DNA methyltransferase activity at each plant developmental stage may be different (Cao and Jacobsen, 2002). Even though similar zebularine concentrations applied to adzuki seedlings were used to treat callus, there was no significant difference between heat-stressed and heat-stressed with 0.5 mM zebularine in callus. According to Emani et al., the application of 5-azacytidine to seedlings and calli of *Sorghum bicolor* (L.) Moench demonstrated that the most suitable concentration of 5-azacytidine (310 μ M) to induce TE expression in seedlings of this species was toxic in the callus (Emani et al., 2002). The optimal 5-azacytidine concentration was instead found to be 20 μ M for the callus in that study. Therefore, the most suitable zebularine concentration for adzuki bean callus induction should be determined in the near future. Indeed, the extent of gene activation may be stronger in the callus than in seedlings because cell division activities are higher in callus tissues, resulting in faster rates of DNA synthesis (Weber et al., 1990). Treatment of plants with concentrations of DNA methylation inhibitors that are too high may lead to cell death because they have cytotoxic effects, such as by causing DNA damage via the covalent trapping of the

DNA methyltransferase rather than DNA demethylation itself (Yamamoto et al., 2005).

Although zebularine was previously reported to be an efficient DNA methylation inhibitor, its effectiveness may be increased further by inhibiting Pol II via α -amanitin (Lindell et al., 1970). This is because the latter process would stabilize the active state of the TE by reducing the production of both its sense and antisense transcripts, which would decrease the accumulation of primary siRNAs, decrease post-transcriptional gene silencing, and increase retrotransposon ecDNA production (Cuerda-Gil and Slotkin, 2016). Moreover, combining zebularine and α -amanitin led to the production of massive amounts of ecDNA, and resulted in the occurrence of 75 novel *ONSEN* insertions per plant in a previous study (Thieme et al., 2017).

In conclusion, the expression levels and production of ecDNA of the heat-activated *ONSEN* retrotransposon in *A. thaliana* and *V. angularis* were found to be increased by treatment with zebularine. However, this increase did not occur in a dose-dependent manner, because each cultivar has its own optimal concentration that led to the highest expression level of this retrotransposon. Our results should be useful in molecular breeding studies, for example to find more precocious strains of plants or those that could more readily adapt to environmental change (Ito et al., 2016). Currently, a method of using inhibitors of retrotransposon silencing exists, called BUNGEE (breeding using jumping genes) (Thieme et al., 2017), which is also useful in the production of non-transgenic crops and can promote molecular breeding by the use of new insertions, such as the retrotransposons examined in the present study.

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