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Research Paper

Characterization of a heat-activated retrotransposon in *Vigna angularis*

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In plants, several transposable elements are conserved across species. We found a homolog of *ONSEN*, which is a heat-activated retrotransposon originally isolated from *Arabidopsis thaliana*, in *Vigna*. The *ONSEN*-like elements (*VaONS*) were detected in all the analyzed Japanese accessions of *Vigna angularis* (adzuki bean) by Southern blot analysis. However, *VaONS* sequences were observed to be polymorphic in the different accessions. Interestingly, extrachromosomal DNA (ecDNA) was detected in some accessions of adzuki bean, indicating the conserved heat-activation of *VaONS*. Furthermore, we successfully induced retrotransposition of *VaONS* in adzuki plant regenerated through callus. Findings of our study should provide a new tool for molecular breeding of adzuki bean.

Key Words: heat stress, adzuki, *Vigna angularis*, *ONSEN*, retrotransposon.

Introduction

Transposable elements (TEs) are abundant in plant and animal genomes and can influence genome evolution and expression of genes (Kazazian 2004, Kumar and Bennetzen 1999, Wessler 1996). TEs are mainly divided into two classes based on their transposition mechanism (Finnegan 1989, Flavell *et al.* 1994). Class-I TEs are retrotransposons and are transposed via an RNA intermediate, by a "copy and paste" mechanism. Class-II TEs involve DNA transposons and are transposed by a "cut and paste" mechanism. Retrotransposons are divided into two families based on the presence or absence of a long terminal repeat (LTR). The LTR retrotransposons are further classified into Ty1/copia-like or Ty3/gypsy-like depending on the order of their coding domains.

Although most TEs are silenced because of epigenetic modifications, such as DNA methylation or histone modification, some TEs are activated under stress conditions (Bennetzen 1987, Chandler and Walbot 1986, Grandbastien 2004, Grandbastien *et al.* 1997, Hashida *et al.* 2003, Henderson and Jacobsen 2007, Hirayama *et al.* 2009, Hirochika 1993, Lisch 2009, Scortecci *et al.* 1997, Steward *et al.* 2000, Zeller *et al.* 2009). Some LTR retrotransposons contain cis-regulatory sequences in their 5′-LTR that can be recognized by a stress-induced transcription factor, which triggers the expression of transposon in response to a particular stimulus (Casacuberta and Gonzalez 2013). The regulatory sequences are variable, suggesting that retrotransposons could have evolved through the modification of regulatory features. Previously, we found heat stress-induced activation of a Ty1/copia-like retrotransposon, *ONSEN*, in *Arabidopsis thaliana* (Ito *et al.* 2011). The activation of *ONSEN* requires a heat-induced transcription factor, HSFA2 (Cavrak *et al.* 2014). HSFA2 binds to a cis-regulatory sequence (heat response element, HRE) in the promoter of *ONSEN* LTR. The activated *ONSEN* is not only transcribed but is also transposed in heat-stressed plants that are deficient in the RNA-directed DNA methylation (RdDM) pathway (Ito *et al.* 2011, Matsunaga *et al.* 2012).

Activation of TEs in response to stress induces mutations that could help the organism adapt to new environmental conditions (McClintock 1984). In *A. thaliana*, *ONSEN* is preferentially inserted within or close to genes (Ito *et al.* 2011) and changes the expression of the flanking genes. Transposition of *ONSEN* was reported to generate a mutation in an abscisic acid (ABA) responsive gene, resulting in an ABA-insensitive phenotype in *A. thaliana* (Ito *et al.* 2016). As such, *ONSEN* family retrotransposons are potential genetic tools for generation of novel phenotypes in host plants.

Ty1/copia-like retrotransposons are ubiquitous in legume crops (Kanazawa *et al.* 2009, Lall *et al.* 2002, Patil *et al.*

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2015, Pearce 2007, Pearce *et al.* 1996, Rajput and Upadhyaya 2010a, 2010b, Sant *et al.* 2000, Wang *et al.* 2004, Xiao *et al.* 2007); therefore, in the present study, we studied whether the *ONSEN* family members are present as functional elements in adzuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi], an important legume crop in Japan. Adzuki bean is one of the major crops in Hokkaido. Intensive efforts are invested in producing new cultivars of this bean. However, small genetic variation in the cultivated adzuki beans has often been a bottleneck in finding the desired traits for cross breeding. Mutagenesis using TEs might be helpful in expanding the genetic diversity of this bean. In addition, it is now easy to find repeat elements in adzuki bean, because a reference-grade genome sequence has become available (Sakai *et al.* 2015).

In this study, in addition to the cultivated adzuki bean, we also included wild adzuki bean, because many accessions are available from NARO Genebank (http://www.gene.affrc.go.jp/index_en.php). The wild adzuki bean is considered to be a progenitor of the cultivated adzuki bean and both are highly cross-compatible with each other (Wang *et al.* 2004). Moreover, because the wild accessions in East Japan have a different karyotype from those in West Japan (Wang *et al.* 2015), we also investigated the correlation between the insertion polymorphisms of *ONSEN* and the geographic origins of the accessions.

Here we report the characterization of a heat-activated *ONSEN* family in the wild and cultivated adzuki bean. We found *de novo* insertions in regenerated plants, indicating that at least one *ONSEN* element can be fully activated in the calli.

Materials and Methods

Identification of ONSEN-like sequences in the Vigna genome

The sequences of transposable elements that are orthologous to the *ONSEN* family in *A. thaliana* (*AtONS*) were identified by BLAST search against available public databases of *Vigna radiata* (Kang *et al.* 2014) and *Vigna angularis* (Sakai *et al.* 2015). The whole genome sequences were downloaded and local BLAST search was performed with the sequence of reverse transcriptase region of *AtONS*. The obtained sequences of the RT region were aligned and a phylogenetic tree was constructed using other COPIA family sequences from *A. thaliana*. The sequences that were orthologous to *AtONS* were retained for further analyses. To obtain full-length sequences of *Vigna ONSEN*-like elements, 10-kb regions on each side, flanking the RT regions, were analyzed using the LTR finder (Xu and Wang 2007). The sequences between the LTRs were aligned to determine the consensus sequence for the reference full-length sequence of each species. Because the consensus sequence from *V. angularis* had a 1-bp insertion, which caused a frameshift in the CDS, the 1-bp insertion was deleted to obtain a full-length reference sequence. The full-length reference sequences were 4895 and 4890 bp in length for *V. angularis* (413 bp LTR) and *V. radiata* (410 bp LTR), respectively. The reference sequences (**Supplemental materials**) were then used for BLAST search against the whole genome sequences to estimate the copy number in each species. The *ONSEN*-like elements in each species were retrieved when the BLAST hits were more than 300 bp in length and were not similar to the sequences of other COPIA family members.

Plant material and growing conditions

A set of 117 accessions of *V. angularis* used for Southern blot analysis was procured from the National Institute of Agrobiological Sciences (Tsukuba, Japan). The accessions were categorized by habitat in East and West Japan (**Supplemental Tables 2**, **3**) based on a previous study by Wang *et al.* (2015). The habitat of cultivated accessions was referred to the habitat of wild accessions so that some cultivated accessions were not assigned (**Supplemental Table 3**). One commercially bred adzuki, *Tanba Dainagon*, was obtained from Takii & Company Limited, Kyoto, Japan. One variety of *V. angularis*, named *Shumari*, was obtained from the Hokkaido Research Organization (Obihiro, Japan). The plants were grown on Murashige and Skoog (MS) plates at 21°C under continuous light conditions.

Southern blot analysis

Genomic DNA was isolated using the Nucleon PhytoPure DNA extraction kit (GE Healthcare Life Science, Chicago, IL, USA). Southern blotting was performed as described previously (Miura *et al.* 2004). The DNA was digested with *Eco*RV and the hybridization signals were detected using a radiolabeled *VaONS*-specific probe (**Supplemental Table 4**) generated using the Megaprime DNA Labeling System (GE Healthcare Life Science), in high sodium dodecyl sulfate hybridization buffer (Church and Gilbert 1984). The independence of the detected bands was statistically tested by Fisher's exact test.

Heat stress treatment

Seven-day-old seedlings grown on MS plates at 21°C under continuous light conditions were subjected to a temperature shift from 4°C for 24 h to 40°C for 24 h. DNA and RNA were immediately extracted from the stressed seedlings after the heat treatment. As a control, seedlings were subjected to a temperature shift from 4°C for 24 h to 21°C for 24 h.

RT-PCR

Total RNA was extracted from the 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). For *VaONS* transcription, RNA was reverse-transcribed using the ReverTraAce qPCR RT Kit (Toyobo, Osaka, Japan) with a random primer.

Polymerase chain reaction was performed using TaKaRA Ex Taq (TaKaRA, Shiga, Japan). The reaction conditions were as follows: 5 min at 94°C; 25 cycles of 94°C (30 sec), 55°C (30 sec), and 72°C (1 min); 7 min at 72°C. As a control, *ACT2* transcript was analyzed by OneStep RT-PCR Kit (QIAGEN, Venlo, Netherlands) using the following reaction conditions: 30 min at 50°C; 15 min at 95°C; 35 cycles of 94°C (30 sec), 55°C (30 sec), and 72°C (1 min); 7 min at 72°C. The sequences of the primers used are summarized in **Supplemental Table 4**.

Phylogenic analysis

The phylogenetic relationships were analyzed using the neighbor-joining method. The synonymous distances were calculated from the reverse transcriptase region (2994 to 3761 and 2991 to 3758 nucleotide positions for reference sequences of *V. angularis* and *V. radiata*, respectively) of the *ONSEN* sequences by the method of Nei and Gojobori. Indel sites were analyzed with pair-wise deletion option. All the analyses were performed with MEGA 7.0 (Kumar *et al.* 2016). The bootstrap probabilities were estimated by 500 replications.

Callus induction

The seedlings of *V. angularis* were grown on MS plate with continuous light at 27°C for 12 days and subsequently subjected to a temperature shift from 4°C for 24 h to 40°C for 24 h. The hypocotyl of a heat-stressed plant was transferred to callus induction medium, consisting of 4.4 g L^{-1} MS with 0.5 g L^{-1} 2-morpholinoethanesulfonic acid (MES), 30 g L^{-1} sucrose, 0.1 mg L^{-1} BA, and 8 g L^{-1} agar.

Results

Characterization of ONSEN in Vigna angularis and V. radiata

About 40 *ONSEN*-like elements were detected in *V. angularis* and *V. radiata* (**Supplemental Table 1**). Of these, three and four copies of *ONSEN*-like elements were solo-LTRs in *V. angularis* and *V. radiata*, respectively. About half of the copies, including these solo-LTRs, were observed to be partial sequences. Even when both the LTRs (partial in some cases) were present, large insertions or deletions were observed, especially in the *ONSEN*-like elements of *V. radiata* that possibly lack mobility. Nine copies in *V. angularis* and six copies in *V. radiata* were full-length copies without large structural variations, although most of them had stop codons or frame-shift mutations because of small indel variations in their CDS regions. In *V. angularis*, only three copies of *ONSEN*-like elements had complete structures, without any disruptive variations (**Fig. 1A**) although no copy had complete structures in *V. radiata*. Because accuracies of genome sequences differ among the species, un-sequenced (un-assembled) regions are expected in the whole genome sequences; this was especially observed for *V. radiata*.

Furthermore, we analyzed the distance between *VaONS* and the flanking gene in *V. angularis*. The average distance was 4187 bp and the distance between the newly inserted *VaONS* that contained conserved LTRs (p-distance < 0.05)

Fig. 1. Structure and phylogenetic relationship of *ONSEN*-like elements in *Vigna* species. (A) The schematic figure of full-length *ONSEN*-like elements in *V. angularis*. GAG: gag polyprotein, PR: protease, INT: integrase, RT: reverse transcriptase, RH: ribonuclease H. probe: A Southern probe designed in the open reading frame (ORF). (B) A phylogenetic tree was made by the neighbor-joining method with synonymous distance estimated by the Nei and Gojobori method. Bootstrap probabilities over 50% are shown along the branches. The copies of *V. angularis* and *V. radiata ONSEN*-like elements are indicated by closed and open labels, respectively. The LTR identities are indicated by different label shapes: large circles >99%, small circles ~99–95%, diamonds ~95–90%, squares <90%, and triangles one or no LTRs. Scale bar is shown on left at the bottom of the figure. *AtONS* copies were included as out-groups. Arrows indicate copies without any disruptive mutations, suggesting that they are active copies.

and the flanking gene was 1048 bp (**Supplemental Table 1**). The distance was shorter than the average distance between the genes in the genome (14 kb: Sakai *et al.* 2015). Indeed, of the 36 *VaONS* sequences, seven were within the gene-coding sequences (**Supplemental Table 1**).

The evolutionary history of *ONSEN*-like elements in *Vigna* species is shown in **Fig. 1B**. For LTR retrotransposons, the sequence divergence within the copies of individual elements between the two LTRs has been proposed as an internal indicator of age, as these are usually identical to each other upon insertion (Kijima and Innan 2010, SanMiguel *et al*. 1996). Several copies had long external branches suggesting old insertion events. These copies showed small LTR identities or a lack of LTR. Recently-amplified copies (more than 95% LTR identities) and several copies made species-specific clusters. The clusters were supported by more than 80% bootstrap values. The presence of species-specific clusters suggests rapid amplification and degradation of *ONSEN*-like elements in *Vigna* species.

Copy number of VaONS

To analyze the copy number variations in *VaONS*, we conducted a Southern blot analysis using the DNA extracted from the accessions of wild and cultivated adzuki beans. About 14 to 16 bands were detected in each accession, of which 12 were common in all the analyzed accessions (**Fig. 2A**, **2B**). The average differences of the number of detected bands between the accessions were 0.886 in the wild accessions and 0.975 in the cultivated accessions. We analyzed the correlation of the band patterns and the origin of

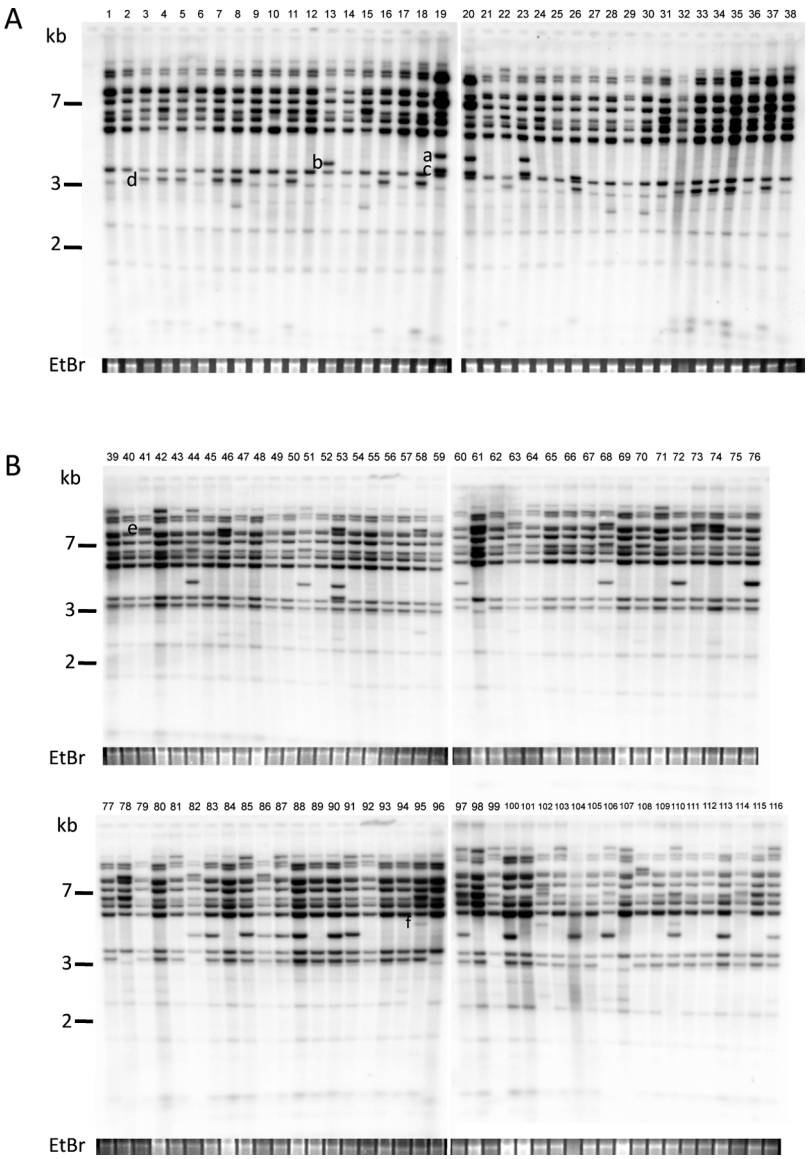

Fig. 2. Variation in the copy numbers of *VaONS* in wild (A) and cultivated (B) adzuki beans. The number on each of the lanes indicates independent adzuki accessions corresponding to that in **Supplemental Tables 2**, **3**. A gel stained with ethidium bromide (EtBr) is shown at the bottom of each panel as a loading control.

Table 1. Relation of polymorphic band and geographical origins

		# of Eastern accessions		# of Western accessions		
Wild accessions	Band (bp)	+	–	+	–	p [1]
	a (5000)	0	9	3	26	0.43
	b (4500)	0	9	1	28	0.76
	c (4100)	0	9	4	25	0.32
	d (3300)	4	5	11	18	0.51
Cultivated accessions	Band (bp)	+	–	+	–	p [1]
	e (9000)	3	13	5	28	0.52
	f (5500)	0	16	2	31	0.45
	a (5000)	3	13	13	20	0.13
	c (4100)	1	15	0	33	0.33
	d (3300)	15	1	28	5	0.35
		# of Wild accessions		# of Cultivated accessions		
Wild vs cultivated	Band (bp)	+	–	+	–	p [1]
	e (9000)	0	38	12	66	0.0063**
	f (5500)	0	38	3	75	0.30
	a (5000)	3	35	22	56	0.0091**
	b (4500)	1	37	0	78	0.33
	c (4100)	4	34	1	77	0.039*
	d (3300)	15	23	71	7	0.00***

The band a to f correspond to the band in **Fig. 2**.

[1] The independence of the detected bands was statistically tested. p-values estimated by Fisher's exact test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

+ or – indicate the presence or absence of the listed band in each accessions.

the accessions (i.e. Eastern vs. Western and Wild vs. Cultivated). Although there were no significant differences between the accessions from the different geographical origins, there were several significant differences between the wild and cultivated accessions (**Table 1**). A 9-kb band was specific to the cultivated accessions. Other two bands at 5 kb and 3.3 kb were also represented more in the cultivated accessions.

Heat activation of VaONS

To examine the heat-activation of *VaONS*, we analyzed the expression of *VaONS* in two varieties of *V. angularis*, namely *Shumari* and *Tanba Dainagon*. The result showed that *VaONS* was activated in both the varieties, when subjected to heat stress (**Fig. 3A**). To detect the presence of an intermediate for transposition of *VaONS*, we conducted Southern blot analysis with non-digested DNA. The ecDNA was detected in the heat-stressed *Tanba Dainagon* but not in *Shumari* (**Fig. 3B**).

Next, we analyzed the ecDNA in the Japanese accessions of wild and cultivated adzuki beans. The ecDNA was detected in 36 out of the 74 (49%) cultivated accessions and in 12 out of the 38 (32%) wild accessions subjected to heat stress (**Fig. 3C**, **3D**, **Supplemental Tables 2**, **3**). To find a relationship between the accession-specific copy and heat-activation, we compared the presence of polymorphic *VaONS* bands detected by Southern blot and the synthesized ecDNA (**Table 2**, **Supplemental Tables 2**, **3**). The result showed that presence of a band specific to the cultivated accessions strongly associated with the ecDNA of *VaONS* (**Fig. 2B**, **Table 2**).

We also analyzed the geographical distribution of the accessions that synthesized the ecDNA, in Japan. In the wild accessions, ecDNA was detected in four out of nine accessions from East and in eight out of 29 accessions from West ($p = 0.29$). In the cultivated accessions, ecDNA was detected in 11 out of 15 accessions from East and in 14 out of 33 accessions from West ($p = 0.046$; **Table 3**). The result showed that a significant number of cultivated accessions that synthesized ecDNA were collected from East Japan.

Detection of new VaONS insertion in tissue culture

To analyze the transposition of *VaONS*, we induced callus from the hypocotyl of heat-stressed *Tanba Dainagon*, which was obtained from a seed company. The callus was subsequently transferred to an appropriate organ-induction medium and a complete plant was regenerated from it. New *VaONS* insertions were detected in the plant regenerated from the callus (**Fig. 4**).

Discussion

On exploring the genome sequences of *V. angularis* and *V. radiata*, about 40 regions were found to be *ONSEN*-related sequences (**Supplemental Table 1**). Phylogenetic analysis and estimation of the insertion age revealed species-specific clusters, comprising of recently transposed copies of the *ONSEN*-related sequences. This could have occurred because the amplification rate was high and the amplification was recent in both the species. However, most of the *ONSEN*-related sequences were observed to be partial fragments and only few regions had the entire sequence of

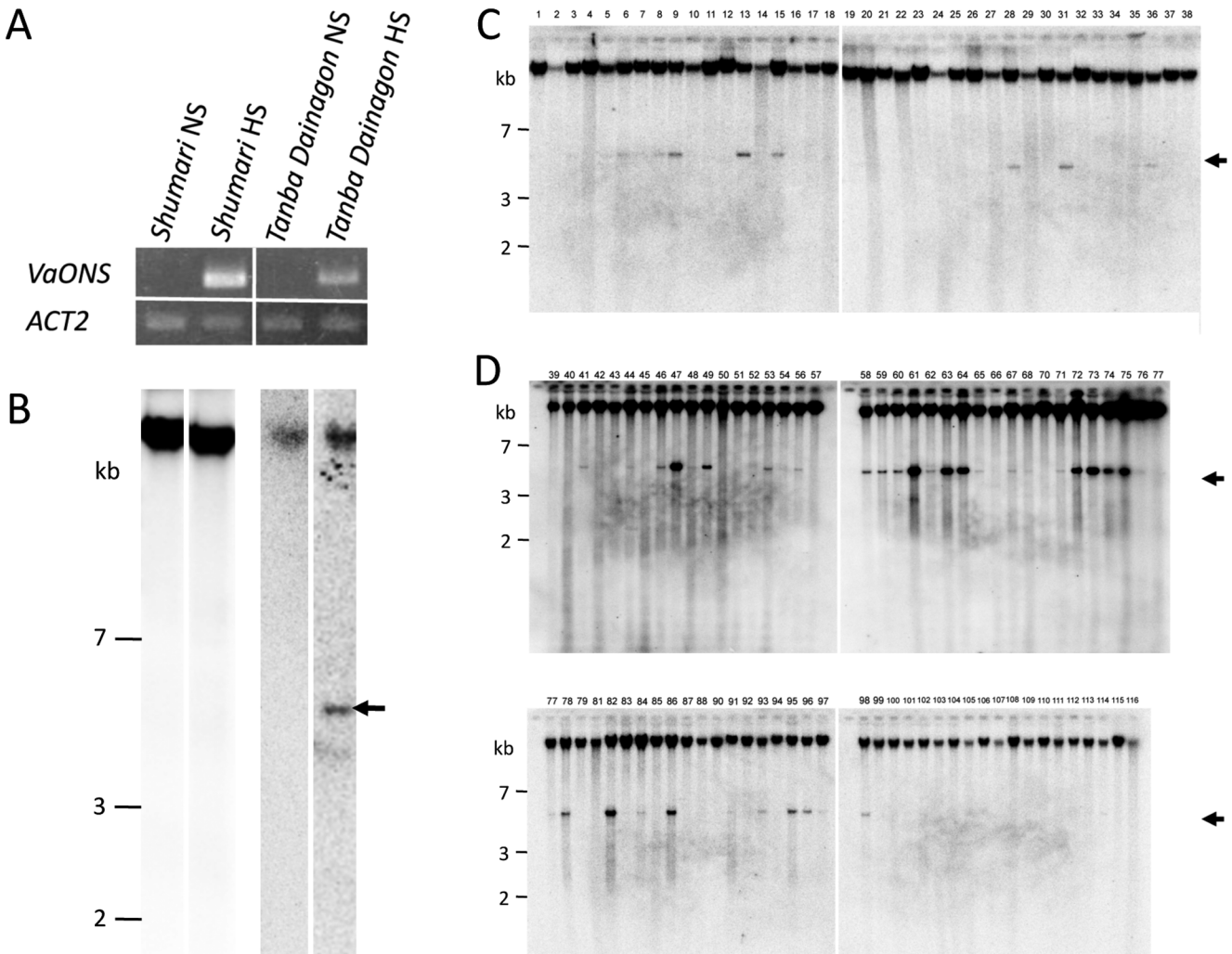

Fig. 3. Effect of heat stress on *VaONS* activation. (A) Heat-induced *VaONS* transcription. Levels of *VaONS* transcripts in the two adzuki varieties subjected to 22°C or 40°C were quantified by RT-PCR, with *ACTIN2* (*ACT2*) transcripts used as an internal control. (B, C, D) Southern blot of non-digested DNA loaded to detect extrachromosomal DNA in the two adzuki varieties (B), and in the wild (C) and cultivated (D) adzuki beans in Japan subjected to heat stress. Arrows indicate extrachromosomal DNA (5 kb) of *VaONS*.

the TE. The genome of *V. angularis* has three complete copies of *ONSEN*-related elements, whereas *V. radiata* has no mobile copies although the presence of active copies is assumed in the non-sequenced genomic regions. The older inserted copies tend to have many disruptive mutations (frameshifts, novel stop codons, and large insertion–deletions). Loss of active copies could cause the elimination of all the family members because there will be no amplification resulting in new copies. A similar situation was observed in *Brassicaceae* species (Ito *et al.* 2013), where *Capsella* and *Cardamine* species had no *ONSEN*-related sequences. This could occur because of the transposition tendencies of the *ONSEN* family as they transpose close to the genic regions of actively transcribed loci (Ito *et al.* 2011). Because the *ONSEN* family sequences transposed to or near the genes in *Vigna* species (**Supplemental Table 1**), the transposition could be deleterious and could effectively be silenced or eliminated. The rapid amplification and elimination cycle caused species-specific clustering of relatively young inserted copies of the *ONSEN*-related sequences (**Fig. 1**). The shorter external branches in *V. angularis*, compared to those in *V. radiata*, also suggest lesser activities in *V. radiata* as was also suggested in simulation studies (Kijima and Innan 2013, Navarro-Quezada and Schoen 2002).

The copy number of *VaONS* was conserved in the Japanese accessions indicating that the retrotransposition activity of *VaONS* was tightly regulated, although it was activated by heat stress. There was no difference of the number of polymorphic bands between East and West varieties although there was significant difference between wild and cultivated accessions. Most polymorphic bands were polymorphic in both the wild and cultivated accessions. Only one and two bands were specific to the wild and cultivated accessions respectively. Because wild adzuki bean is often found around the fields of farmers, outcrossing occurs at around 1% (Yamamoto *et al.* 2006). Our results also support

Table 2. Relation of polymorphic band and ecDNA

		# of Accessions with ecDNA		# of Accessions without ecDNA		
Wild accessions	Band (bp)	+	–	+	–	p [1]
	a (5000)	0	12	3	23	0.31
	b (4500)	1	11	0	26	0.32
	c (4100)	0	12	4	22	0.2
	d (3300)	5	7	10	16	0.56
Cultivated accessions	Band (bp)	+	–	+	–	p [1]
	e (9000)	11	25	1	37	0.0011**
	f (5500)	1	35	2	36	0.52
	a (5000)	10	26	12	26	0.46
	c (4100)	1	35	0	38	0.49
	d (3300)	32	4	35	3	0.47
All accessions	Band (bp)	+	–	+	–	p [1]
	e (9000)	11	25	1	37	0.00034***
	f (5500)	1	35	2	36	0.61
	a (5000)	10	38	15	49	0.46
	b (4500)	1	11	0	26	0.42
	c (4100)	1	47	4	60	0.28
	d (3300)	37	11	45	19	0.28

[1] The independence of the detected bands was statistically tested. p-values estimated by Fisher's exact test. **: $p < 0.01$, ***: $p < 0.001$.
+ or – indicate the presence or absence of the listed band in each accessions.

Table 3. Relations of geographical origin and *VaONS* ecDNA

	Eastern accessions		Western accessions		
Presence of ecDNA	+	–	+	–	p [1]
Wild accessions	4	5	8	21	0.29
Cultivated accessions	11	4	14	19	0.046*
	Wild accessions		Cultivated accessions		
Presence of ecDNA	+	–	+	–	p [1]
Wild vs cultivated	12	26	25	23	0.045*

[1] p-values estimated by Fisher's exact test. *: $p < 0.05$.
+ or – indicate the presence or absence of ecDNA in each accessions.

the ongoing introgression between the wild and cultivated adzuki beans in Japan.

The heat activation of *ONSEN* was originally observed in *A. thaliana* and we previously reported the heat-activation of *ONSEN*-like elements among *Brassica* species (Ito *et al.* 2013). In this report, we analyzed the heat-activation of the element in more distant dicot relatives, the adzuki beans. We found that the heat activation was conserved in adzuki species and some *ONSEN* copies synthesized the ecDNA. As is evident from the sequence analysis, three *VaONS* copies had complete structure and could be activated by heat stress in adzuki beans. Southern blot analysis showed that more ecDNA was detected in the accessions from East than in those from the West, even among the genetically homogeneous cultivated accessions, suggesting that the gene flow of active copies of *VaONS* between the cultivated and wild accessions had occurred in adzuki beans (Wang *et al.* 2004).

The ecDNA was detected in the seedlings subjected to 40°C for 24 h; however, the DNA was not detected in the seedlings subjected to 37°C for 24 h (data not shown). This indicated that the temperature which was sufficient to activate the *ONSEN*-like elements was variable among the species and *VaONS* might be less sensitive to heat stress compared to the element in *A. thaliana*. It is worth mentioning that *VaONS* was expressed in two varieties of *V. angularis*, namely *Shumari* and *Tanba Dainagon*; however, the ecDNA was detected only in *Tanba Dainagon*. The *ONSEN*-like retrotransposons that have stop codons or frame-shift mutations in the CDS regions could be transcribed by heat stress, because the expression of the retrotransposon was regulated by a heat-shock transcription factor that was bound to HRE in the LTR promoter. Our results indicated that the transcriptional activation and the retrotransposition *via* ecDNA were not always associated.

As is possible for many other TEs (de Araujo *et al.* 2005, Hirochika 1993, Hirochika *et al.* 1996, Madsen *et al.* 2005, Masuta *et al.* 2017, Planckaert and Walbot 1989, Sato *et al.* 2011, Yamashita and Tahara 2006, Yilmaz *et al.* 2014), we could successfully induce retrotransposition of *VaONS* by tissue culture (**Fig. 4**). It requires further research to understand the mechanism of the callus-mediated transposition of *ONSEN*; however, we have reported that the transposition of heat-activated *ONSEN* during tissue culture was regulated by an alternative mechanism in addition to the RdDM-mediated epigenetic regulation in *A. thaliana* (Masuta *et al.* 2017). Although mutagenesis *via* culture-induced transpositions has problems, including the presence of somaclonal variations in the regenerated plants (Bairu *et al.* 2011, Larkin and Scowcroft 1981) and requires relatively costly manipulation, it does generate new alleles and would help in expanding the genetic variation of adzuki bean.

To conclude, our results demonstrate that the *ONSEN*-like elements (*VaONS*) were present in all the analyzed Japanese accessions of adzuki bean (*Vigna angularis*), as

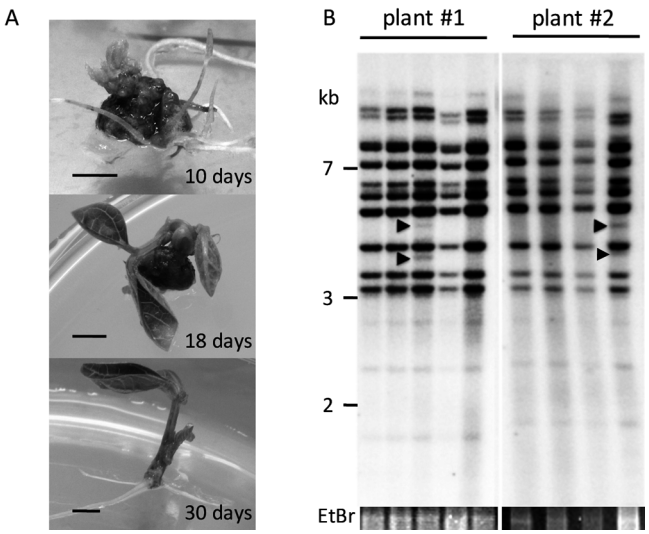

Fig. 4. Callus-mediated transposition of *VaONS* in regenerated adzuki plant. (A) Callus induction and dedifferentiation in *Vigna angularis*. The days indicate the duration of feeding period after the callus induction. Scale bar indicates 5 mm. (B) Southern blot of *VaONS* in the regenerated plants. Each lane represents the regenerated individuals derived from two independent plants. Arrowheads indicate new insertions of *VaONS*. A gel stained with ethidium bromide (EtBr) is shown at the bottom of each panel as a loading control.

detected by Southern blot analysis. We found that the heat activation of *ONSEN* copies was conserved in adzuki species and some *ONSEN* copies synthesized the ecDNA. Moreover, we could successfully induce retrotransposition of *VaONS* by tissue culture. Adzuki bean cultivars are always challenged by cold stress, new types of bacterial diseases, and nematode infections. Our findings should shed light on new materials that could be useful in molecular breeding of resistant varieties. Because *VaONS* is functional in adzuki bean, there might be other active TEs in this and related species. Given the fact that many other species of *Vigna* are being sequenced (Sakai *et al.* 2015), it would be worth trying to find such elements for further understanding of genome evolution and for using them as a tool in breeding of new varieties of plants.

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