

A *Stowaway* transposon disrupts the *InWDR1* gene controlling flower and seed coloration in a medicinal cultivar of the Japanese morning glory

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(Received 1 September 2015, accepted 10 November 2015; J-STAGE Advance published date: 12 April 2016)

Floricultural cultivars of the Japanese morning glory (*Ipomoea nil*) carry transposons of the *Tpn1* family as active spontaneous mutagens. Half of the characterized mutations related to floricultural traits were caused by insertion of *Tpn1* family elements. In addition, mutations comprising insertions of several bp, presumed to be footprints generated by transposon excisions, were also found. Among these, *ca-1* and *ca-2* are 7-bp insertions at the same position in the *InWDR1* gene, which encodes a multifunctional transcription regulator. *InWDR1* enhances anthocyanin pigmentation in blue flowers and red stems, and promotes dark brown seed pigmentation as well as seed-trichome formation. The recessive *ca* mutants show white flowers and whitish seeds. We characterized here a white flower and whitish seed line that is used as a medicinal herb. The mutant line carries a novel *ca* allele named *ca-3*, which is the *InWDR1* gene carrying an insertion of a *Stowaway*-like transposon, *InSto1*. The *ca-3* allele is the first example of a mutation induced by transposons other than those in the *Tpn1* family in *I. nil*. Because *InSto1* and the 7-bp putative footprints are inserted at identical positions in *InWDR1*, *ca-3* is likely to be the ancestor of *ca-1* and *ca-2*. According to Japanese historical records on whitish seeds of *I. nil*, putative *ca* mutants appeared at the end of the 17th century, at the latest. This is around one hundred years before the appearance of many floricultural mutants. This suggests that *ca-3* is one of the oldest mutations, and that its origin is different from that of most floricultural mutations in *I. nil*.

Key words: anthocyanin, flower pigmentation, *Ipomoea nil*, *Tc1/Mariner*, transposon

The Japanese morning glory (*Ipomoea nil*) is a traditional floricultural plant in Japan. A number of its spontaneous mutants have been isolated since the early 19th century (Imai, 1927; Yoneda, 1990; Iida et al., 2004). Most of the mutants exhibit alterations of floricultural traits in flower and leaves. Recent studies revealed that transposons in the *Tpn1* family cause a large proportion of the *I. nil* mutations. The *a3^f*, *sp*, *r1*, *pr* and *efp* mutations related to flower coloration, and the *dp* and *fe* mutations involving leaf and flower shapes, are caused by the insertion of *Tpn1* family elements (Inagaki et al., 1994;

Fukada-Tanaka et al., 2000; Hoshino et al., 2001, 2009; Nitasaka, 2003; Iwasaki and Nitasaka, 2006; Morita et al., 2014). These elements are DNA transposons and belong to the *En/Spm* or *CACTA* superfamily (Inagaki et al., 1994; Kawasaki and Nitasaka, 2004). DNA transposons including *Tpn1* family elements sometimes generate mutable alleles that spontaneously show recurrent somatic and germinal mutations.

DNA transposons generate target site duplication (TSD) upon their integration. They often leave small DNA rearrangements, called footprints, at their excision sites. Footprints sometimes include sequences of duplicated target sites. The length of the TSD of the *Tpn1* family elements is 3 bp. Among the mutations in *I. nil*, several-bp insertions have been found repeatedly. The

Edited by Hiro-Yuki Hirano

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DOI: <http://doi.org/10.1266/ggs.15-00062>

dy, *ca* and *dk* mutations are 4-bp (GGAT and CGAT in *dy*-1 and *dy*-2, respectively), 7-bp (GGAGTAC and TCCG-TAC in *ca*-1 and *ca*-2, respectively), and 4-bp (GGCC in *dk*-1) insertions (Morita et al., 2005, 2006, 2015). The 3'-most 3-bp sequences of the presumed insertion sequences are identical to the 3-bp sequences adjacent to their 5' ends. These sequence features suggested that they were footprints of *Tpn1* family elements. Thus far, the transposons generating these putative footprints have not been identified.

In the seed stock made by Nariyasu Shimazu of Ibaraki University, a line named Yakuyou-shirohana was collected. In Japanese, the name Yakuyou means medicinal. *I. nil* was introduced into Japan from China as a medicinal herb, and its seeds have been used as laxatives. In addition to wild-type black seeds, whitish seeds also have long been known as laxatives. Yakuyou-shirohana shows white flowers, green stems and whitish seeds (Fig. 1), while wild-type plants produce blue flowers, red-pigmented stems and black or dark brown seeds. From these phenotypes, we hypothesized that the line carries a *ca* mutation. The *Ca* gene, also called the *InWDR1* gene, encodes a WD40 repeat protein (Morita et al., 2006). It activates anthocyanin pigmentation in flowers and stems, and is essential for normal seed pigmentation by proanthocyanidins and phytomelanins and for seed-trichome formation (Morita et al., 2006; Park and Hoshino, 2012).

To characterize the *InWDR1* gene in Yakuyou-shirohana, we employed PCR analysis. Genomic DNA was isolated from flower petals using an automated DNA isolation system, NA-2000 (Kurabo, Osaka, Japan). *InWDR1* fragments that included the whole *InWDR1* coding sequence were amplified using the primers CAKPN-F1 (CCGGGG-TACCGGATCCCATGCATCCAGTGT) and CAKPN-R1 (GCGCGGTACCTACTGGGAGGTGGACTGTCT) (Park and Hoshino, 2012). The fragment from the mutant was approximately 4.5 kb long—about 800 bp longer than that of the wild-type plant—indicating that the mutant carries an insertion sequence (data not shown). To characterize the insertion further, the PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN,

Hilden, Germany) and were directly sequenced on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The insertion sequence was found to be a *Stowaway*-like transposon, which we named *InSto1* (Fig. 2A). *Stowaway* elements are miniature inverted-repeat transposable elements (MITEs) related to the *Tc1/Mariner* superfamily (Bureau and Wessler, 1994; Feschotte et al., 2002, 2003). MITEs are short non-autonomous class II elements in eukaryotic genomes (Feschotte et al., 2002). Although *Stowaway* elements are abundant in various plant genomes, very few *Stowaway* insertion mutations that affect the visible phenotype have been reported. In the potato, a *Stowaway* insertion within the gene that encodes flavonoid 3',5'-hydroxylase resulted in alteration of the tuber anthocyanin coloration (Momose et al., 2010). These elements have a preference for insertion at the dinucleotide TA. *InSto1* is 849 bp long and is inserted within the unique exon of the *InWDR1* gene. The element is flanked by a TSD of the dinucleotide TA. It has an 11-bp terminal inverted repeat (TIR) sequence (Fig. 2B) that shows high similarity with the conserved TIR sequence of *Stowaway* elements (Fig. 2C; Feschotte et al., 2002). A BlastX search revealed that *InSto1*



Fig. 1. Phenotypes of the *ca*-3 mutant Yakuyou-shirohana: flower (A) and seeds (B).

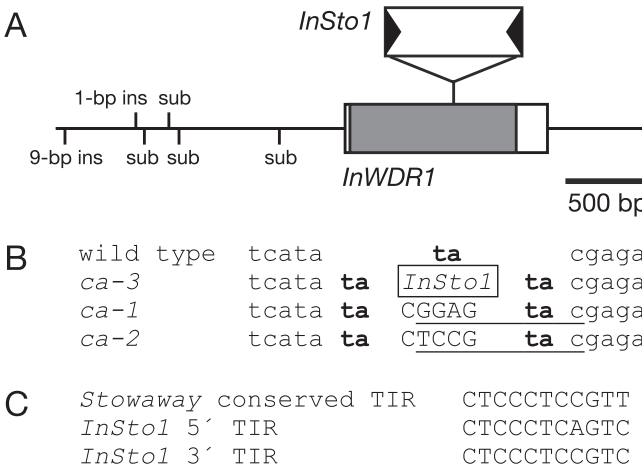


Fig. 2. Characterization of the *ca*-3 allele. (A) Structure of the 4.5-kb PCR fragment containing *InWDR1* in the *ca*-3 mutant. The larger box indicates the unique exon of *InWDR1*; the coding region is shaded gray. The box with filled arrowheads shows *InSto1*. Vertical bars indicate the positions of polymorphisms between the *ca*-3 mutant and a wild-type line, Tokyo Kokei Standard: substitutions and 9- and 1-bp insertions are indicated as sub, 9-bp ins and 1-bp ins, respectively. (B) Sequences of the insertion site of *InSto1* and the putative footprints in *ca*-1 and *ca*-2 alleles. The TA dinucleotide target site is indicated in boldface. The 5-bp insertion sequences putatively created upon *InSto1* excision are shown in uppercase. The underlined 7-bp sequences were previously thought to be footprints of the *Tpn1* family element (Morita et al., 2006) because their 3'-most 3-bp sequences (TAC) are identical to the 3-bp sequences immediately upstream of the putative footprint sequences. (C) Comparison of the conserved TIR sequence of *Stowaway* elements and the *InSto1* TIRs.

carries no coding sequences for transposases or known proteins. This strongly suggests that *InSto1* is a non-autonomous element. We designated the *InWDR1* allele with the *InSto1* insertion as *ca-3*.

Interestingly, the insertion site of *InSto1* is 1 bp upstream to that identified for the 7-bp insertions in the *ca-1* and *ca-2* alleles (Fig. 2B). The original interpretations of the 7-bp insertion sites were therefore presumably wrong, and the 7-bp sequences (CGGAGTA and CTCCGTA in *ca-1* and *ca-2*, respectively) that share an identical insertion site with *InSto1* represent the correct insertions. The newly interpreted 7-bp insertions have 2 bp of TA at their 3' end. This strongly suggests that *InSto1* generated the 7-bp insertions as footprints, the wild-type sequence TA being changed into TA(CGGAG/CTCCG)TA upon *InSto1* excision. In other words, *ca-3* was a precursor of *ca-1* and *ca-2*. Consistent with this notion, Yakuyou-shirohana and a *ca-1* line (NS/W1*ca1*) had identical *InWDR1* sequences except for the *InSto1* insertion site, while several polymorphisms were found between the 5' upstream regions of Yakuyou-shirohana and a wild-type line, Tokyo Kokei Standard (Fig. 2A). The 5' upstream regions in a second *ca-1* (NS/W2*ca3*) and six *ca-2* lines have not been sequenced. In contrast, the 4-bp insertions in the *dy-1*, *dy-2* and *dk-1* alleles cannot be footprints of *InSto1* or related *Stowaway* elements because the 4-bp insertions have no TA dinucleotide at their 3' ends.

According to our survey of Japanese historical literature, the first reliable description of whitish seeds of *I. nil* appeared in an agricultural book, *Nogyo Zensho*, published in 1697 (Miyazaki and Kaibara, 1697). The oldest picture of whitish seeds was printed in an encyclopedia, *Wakan Sansai Zue* (Terashima, 1712). Therefore, a mutation that confers whitish seeds was isolated at the end of the 17th century, at the latest. This is more than a hundred years before the appearance of most of the other *I. nil* mutants. All nine lines showing whitish seeds characterized thus far have been found to carry one of the recessive *ca* mutations, *ca-1*, *ca-2* or *ca-3* (Morita et al., 2006, this study). Therefore, the mutants described in *Nogyo Zensho* and *Wakan Sansai Zue* probably had *ca-3* or its derivatives, and *ca-3* would thus be one of the oldest mutations in *I. nil*. In other words, the origin of the *ca-3* mutation is likely to have been different from that of most mutations related to floricultural traits. Floricultural mutations were assumed to have been isolated from lines carrying active *Tpn1* family elements in Japan (Iida et al., 2004). Whitish seeds of *I. nil* have also been used as a medicinal herb in China for a long time. It can be hypothesized that mutants carrying *ca-3* were first isolated in China and were introduced into Japan. Consistent with this hypothesis, the *ca-3* allele was found in a Chinese traditional herbal line, Baichou. Wang et al. (2013) found a mutant allele of the *InWDR1*

gene from Baichou. While no details of the allele were reported, the sequence of the allele was deposited in GenBank (accession number KF384189) as the *WDR1-sino* allele. Sequence comparison revealed that *WDR1-sino* and *ca-3* are the same allele, although *InSto1* is annotated as a *Tpn*-like transposon rather than a *Stowaway* transposon in the GenBank record.

No evidence indicating the transposition activity of *InSto1* could be detected. No fragments derived from footprints were amplified, even by nested PCR (data not shown). Interestingly, approximately 300 years ago, it was reported that blue sectors were occasionally generated in white flower petals in a whitish seed line, Ginsen (Mimura, 1723). Ginsen possibly carried the *ca-3* allele. If this is the case, *InSto1* occasionally transposed in that period and caused somatic reversions of the *InWDR1* gene, resulting in the appearance of chimeric blue-colored sectors on white petals. We are currently screening *ca-3* lines for active *InSto1* and searching for *InSto1*-related autonomous elements.

The authors thank Seiko Watanabe, Ryoko Nakamura, Tomoyo Takeuchi and Kazuyo Ito for their technical assistance, along with the NIBB Model Plant Research Facility and the NIBB Functional Genomics Facility for their technical support. The nucleotide sequences reported in this paper are available in the DDBJ/EMBL/GenBank databases under accession number LC075770 (the *ca-3* allele) and LC075771 (*InSto1*).

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