Miniature inverted-repeat transposable elements of *Stowaway* are active in potato

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Active *Stowaway* MITEs in potato

MITE, transposon, somaclonal variation

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Miniature inverted-repeat transposable elements (MITEs) are dispersed in large numbers within the genomes of eukaryotes although almost all are thought to be inactive. Plants have two major groups of such MITEs: Tourist and Stowaway. Mobile MITEs have been reported previously in rice but no active MITEs have been found in dicotyledons. Here, we provide evidence that Stowaway MITEs can be mobilized in the potato and that one of them causes a change of tuber skin color as an obvious phenotypic variation. In an original red-skinned potato clone, the gene encoding for a flavonoid 3’,5’-hydroxylase, which is involved in purple anthocyanin synthesis, has been inactivated by the insertion of a Stowaway MITE named dTstu1 within the first exon. However, dTstu1 is absent from this gene in a purple somaclonal variant which was obtained as a regenerated plant from a protoplast-culture of the red-skinned potato. The color change was attributed to reversion of flavonoid 3’,5’-hydroxylase function by removal of dTstu1 from the gene. In this purple variant another specific transposition event has occurred involving a MITE closely related to dTstu1. Instead of being fossil elements, Stowaway MITEs, therefore, still have the ability to become active under particular conditions as represented by tissue culturing.

COLOR mutation or variegation of grain, flower petals or fruit skin represent suitable visual markers for the identification of both genes for pigment production and
transposable elements (CLEGG and DURBIN 2000; WINKEL-SHIRLEY 2001; KOBAYASHI et al. 2004). Recent large scale genome analyses have uncovered numerous transposable elements occupying large portions of eukaryotic genomes. Approximately 45% of the human genome is composed of sequences originating from over three million copies of transposable elements (INTERNATIONAL HUMAN GENOME SEQUENCING CONSORTIUM 2001). Even in rice, a plant with a relatively small genome, 20% of the genomic sequence can be derived from transposable elements (TURCOTTE et al. 2001; GOFF et al. 2002; YU et al. 2002). Although almost all of these insertions are thought to be inactive, these elements are suggested to have influenced the evolution of genomes and individual genes. They can rearrange a genome through transposition, insertion, excision, chromosome breakage or ectopic recombination (BENNETZEN 2000). Moreover, some can contribute to the emergence of a novel gene by conveying a poly(A) signal, transcription start site, TATA box, splicing site or intron (OKI et al. 2008).

Bioinformatic analyses using data of genome projects found miniature inverted-repeat transposable element (MITE) (BUREAU and WESSLER 1992, 1994), the copy number of which reaches over thousands in a genome (FESCHOTTE et al. 2002). Characteristically, a MITE is not longer than 600 bp, does not contain any coding sequences, has imperfect terminal inverted repeats (TIRs) at the end of the element and its target site is duplicated upon insertion. The majority of MITEs in plants are divided into two groups, Tourist and Stowaway, based on the sequences of TIRs and their target
Tourist MITEs are found in grasses while Stowaway is not only present in monocotyledonous but also in dicotyledonous plants (BUREAU and WESSLER 1992, 1994; FESCHOTTE et al. 2002). Although huge numbers of MITEs of each family have been found since their discovery in silico, their dynamic features remain largely unknown. The first mobile MITE, mPing, was identified in rice and belongs to the Tourist family. Its movement was activated during long-term cell culture (JIANG et al. 2003) and by anther culture (KIKUCHI et al. 2003). When mPing was inserted into the gene for rice ubiquitin-related modifier-1 (Rum1), its excision resulted in reversion of the mutable slender glume phenotype to wild type (NAKAZAKI et al. 2003). The identification of an active element made it possible to discover that the transposable elements Ping and Pong supplied the transposase acting on mPing (YANG et al. 2007). Movement of Stowaway MITEs in rice was also reported recently. These were mobilized in yeast cells by transposases of Mariner-like elements (MLEs) (YANG et al. 2009). Active copies of MITEs have been found only in rice. In dicotyledons the only indication that they can be mobilized has come from insertional polymorphisms between accessions or cultivars (MACAS et al. 2005; MENZEL et al. 2006).

How a transposable element becomes active is an interesting question since it is potentially an endogenous mutagen and could represent a force for evolution through rearrangement of a genome or production of novel genes. Cell culture is known to activate transposable elements. For example, Ac and Spm/En of class II (DNA) elements were mobilized under such conditions (PESCHKE et al. 1987; PESCHKE and PHILLIPS
1991) and tissue culturing resulted in a vast increase of copy number of retrotransposons belonging to class I (RNA) elements (GRANDBASTIEN et al. 1989; HIROCHIKA 1993). The activation of transposable elements by culture can cause genetic and phenotypic variation in clonal plants, which is one of the reasons for somaclonal variation (LEE and PHILLIPS 1988; KAEPPLER et al. 2000).

The active Stowaway MITEs reported here induced somaclonal variation and provide a tool to investigate how MITEs have propagated to become a major component of the plant genome and under which conditions they become active.

**MATERIALS AND METHODS**

**Plant Materials:** A commercial triploid potato cultivars named “Jaga kids purple” (‘JKP’) and “Jaga kids red” (‘JKR’) were developed by Kirin Brewery Co., Ltd. (Japan) from leaf protoplasts of a red-skinned, triploid clone ‘72218’, which was obtained by a cross between a tetraploid cultivar “Early rose” (*Solanum tuberosum*) and a diploid related species *Solanum phureja* (TOMIDA and KAWAKAMI 1989). Tubers of ‘72218’, generally designated “Neo-delicious” or “Akadake”, were kindly provided by Dr. Kazuyoshi Hosaka of Kobe University.

**Pigment analysis:** Pigment was extracted from tuber skin with 50 ml 50% (v/v) acetic acid. After filtration, 200 ml of water was added to the extract and this solution was passed over an ODS resin column (Wakosil 25C18, i.d. 15 x 100 mm; Wako Pure Chemical Industries Ltd., Osaka, Japan) equilibrated with aqueous 10% (v/v) acetic acid.
The column was washed with 10% acetic acid, and the fraction with anthocyanins was eluted by methanol containing 0.1% hydrochloric acid. The eluate was dried and the residue was separated by mass TLC (TLC Cellulose (10 x 10 cm); Merck KGaA, Dermstadt, Germany) using TBA (t-buthanol:acetic acid:water = 3:1:1) as the solvent. The anthocyanins, migrating as a colored band, were cut out and extracted by methanol containing 0.1% hydrochloric acid. After evaporation of the solvents, the anthocyanin was dissolved in 1 ml of 1% hydrochloric acid. An equal volume of concentrated hydrochloric acid was added and the solution was heated at 100°C for 20 min to release the anthocyanidins which were extracted by isoamyl alcohol. Anthocyanidins in the resulting isoamyl alcohol layer were identified by HPLC/MS analysis; HPLC/MS (1525 Binary HPLC Pump, 996 Photodiode Array Detector, 2767 Sample Manager, Micromass ZQ; Waters Co., Milford, MA) was equipped with a Synergi 4 m Fusion-RP 80 Å column (4.6 x 100 mm, Phenomenex, Torrance, CA) operated at 30°C. The mobile phase consisted of 1% aqueous formic acid as solvent A and methanol as solvent B, and the gradient program was 20% B to 70% B (20 min) and 100% B isocratic (10 min) at a flow rate of 1 ml/min.

**Southern blot analysis:** Genomic DNA was isolated from the leaves by Nucleon Phytopure Genomic DNA extraction kit (GE Healthcare, Uppsala, Sweden). Approximately 10 µg of genomic DNA was digested with EcoRV then separated by 1% agarose gel electrophoresis. The DNAs were transferred to Hybond N+ (GE Healthcare, Uppsala, Sweden) and then hybridized to PCR-amplified cDNA for F3’5’H::rev as a
probe. Probe labeling and the signal detection were carried out with AlkPhosDIRECT (GE Healthcare, Uppsala, Sweden).

**PCR primers and the reaction condition for cDNA and genomic DNA analyses:**

PCR primers used in this study are listed in supporting information Table S1 with their approximate positions shown in Figure S1. Most PCR reactions were carried out nested, with two primer sets, in order to increase specificity and yield. Each PCR consisted of an initial denaturation step at 95°C for 3 min, followed by thirty cycles at 95°C for 30 sec, 56°C for 30 sec and extension at 72°C for 2 or 5 min with a final 3 min extension at 72°C. Gel-purified PCR products using MagExtract or (Toyobo Co. Ltd., Shiga, Japan) were sequenced directly or after cloning into pCR 4-TOPO using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

**Isolation and sequence determination of the cDNAs for F3'5'H gene:** Total RNA was isolated from approximately 100 mg of tuber skin by using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). In order to obtain the sequence of the cDNA for flavonoid 3',5'-hydroxylase (F3'5'H) gene of ‘JKP’, 5' RACE experiment was performed using GeneRacer kit (Invitrogen, Carlsbad, CA) with supplied and gene specific primers (No. 1 (5'-AACATTTTTGTCAATAAAKCATCAAA-3') and No. 2 (5'-CCTTGTAAATCCATCCAAGCTA-3') for the 1st and the 2nd amplifications, respectively) that anneal to two highly conserved region among P450 or F3'5'H genes of *Solanum melongena* (GenBank accession X70824) (TOGURI *et al.* 1993) and *Petunia*
hybrida (GenBank accession Z22544, Z22545 and X71130) (HOLTON et al. 1993; TOGURI et al. 1993). The gene specific primers for 3' RACE (No. 3 (5'-CCGAATTCAAGCTTTATATTATCTTCGATTTT-3') for the 1st and No. 4 (5'-GGCATTACGTATTAGTGAGTTG-3') for the 2nd amplification) were based on the sequence obtained by the 5' RACE experiment. The outcome of both RACE experiments enabled the design of primers (No. 5 (5'-CCTTCTACTTCATTCTCACTCT-3') and No. 6 (5'-AGCAAATATGTTGCACTATAAATG-3') for the 1st, No. 3 and 6 for the 2nd amplification) to amplify the full-length cDNAs for the \textit{F}3'5'H gene by RT-PCR using 1st strand cDNAs prepared from ‘72218’, ‘JKR’ and ‘JKP’ as templates. The extension time for all PCRs was 2 min.

\textbf{Isolation and sequence determination of the genomic DNA for F}3'5'H \textbf{genes:}

Genomic DNA was isolated from approximately 100 mg of leaves as described previously (WALBOT and WARREN 1988). Genomic DNA of \textit{F}3'5'H gene was amplified (using a 5 min extension time) with primers of No. 5 and 6. The methods for the isolation of the other \textit{F}3'5'H pseudo-genes, \textit{f}3'5'h2 and \textit{f}3'5'h3, are described in the supporting information.

\textbf{Isolation of \textit{dTstu1-2} and the sequence determination proximal to the insertion site in ‘JKP’:} PCR with a primer specific for the internal sequence of \textit{dTstu1} (No. 25 (5'-ATTCATTTTGACCACAAGTTTTA-3')) yielded a ‘JKP’ specific product of 2.5 kb that enabled the design of two new primers (No. 26 (5'-TGTTTTTTTCGAGTTATTTTAC-3') and No. 27
(5’-CAAGGGGAGACATTTAGG-3’)). Inverse PCR on Mbol-digested ‘JKP’ genomic DNA followed by self-ligation (primers No. 26 and No. 27 for the 1st and No. 26 and No. 28 (5’-AGACATTTCATAGGCAAATTGTTA-3’) for the 2nd PCR) produced a ‘JKP’ specific ca. 1 kb fragment containing the flanking sequences of the dTstu1-2 insertion. Here, primer No. 28 was designed from dTstu1 internal sequence. PCR with primers of No. 29 (5’-AGCTGAAATATGAGATTGAAATTAG-3’) and No. 30 (5’-ATTTTGCTATATCCACAATGACTT-3’) annealing to these flanking regions amplified the dTstu1-2 insertion locus from genomic DNAs of ‘72218’ and ‘JKP’. The extension time of all PCR reactions was 5 min.

**MITE display:** Transposon display was carried out using primers designed from the sequence of dTstu1 and dTstu1-2 according to the procedure of CASA et al. 2000. Approximately 250 ng of genomic DNA was digested with MseI and ligated to an adaptor. Aliquots of the reactions were diluted 4-fold with 0.1 x TE. Preselective amplification was performed with a primer complementary to the adapter (Mse+0 (5’-GACGATGAGTCCTGAGTAA-3’)) and another primer complementary to an internal dTstu1 and dTstu1-2 sequence (No. 31 (5’-CATTCTTTTTGGGACTGACTA-3’)). PCR consisted of twenty five cycles at 94°C for 30 sec, 56°C for 30 sec and extension at 72°C for 1 min with a final 5 min extension at 72°C. Aliquots of the reactions were diluted 20-fold with 0.1 x TE. Selective amplification was carried out with a selective primer (Mse+N (5’-GACGATGAGTCCTGAGTAA+N-3’)) and another primer specific for the TIR and target site duplication (TSD) sequence of dTstu1 and dTstu1-2 (No.32
(5'-ATAAAWTGGGACRGAGGGAGTA-3')). The latter primer was labeled at the 5'-end with 6-FAM. Temperature cycling conditions were 94°C for 5 min, ten touchdown cycles of 94°C for 30 sec, 66°C for 30 sec (-1°C each cycle) and extension at 72°C for 1 min, followed by twenty five cycles of 94°C for 30 sec, 56°C for 30 sec and extension at 72°C for 1 min with a final 5 min extension at 72°C. The products were analyzed on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

RESULTS

**Key enzyme of the color variation:** ‘Jaga kids purple’ (‘JKP’) is a potato cultivar with purple tubers which was obtained as a somaclonal variant of skin color after selection from plants regenerated from leaf protoplasts of clone ‘72218’ with red tubers (Figure 1A) (OKAMURA 1991, 1994). Analysis of the anthocyanin aglycones revealed that the crucial difference between these purple and red potatoes was the presence of petunidin in the tuber skin of ‘JKP’ as one of the major anthocyanidins, whereas in ‘72218’ this was pelargonidin. The difference between petunidin and pelargonidin is the number of hydroxyl and methoxyl groups at the B-ring of these molecules. Addition of two hydroxyl groups to dihydrokaempferol, which is the precursor of pelargonidin, produces dihydromyricetin, a precursor of petunidin. This reaction is catalyzed by flavonoid 3’,5’-hydroxylase (F3’5’H) (Figure 1B). Therefore, the cause of the color variation from red (‘72218’) to purple (‘JKP’) was attributed to gain of F3’5’H function in the tuber skin of ‘JKP’. Recovery of F3’5’H gene itself would most likely explain the restoration of
enzyme activity since genetic analysis had revealed that the dominant allele for $F3'5'H$ in
the $P$ locus is solely responsible for determination of the purple color phenotype (JUNG
et al. 2005).

**Analysis of $F3'5'H$ genes:** The possibility that disruption of the $F3'5'H$ gene of
‘72218’ was involved in the coloration of its in tuber skin was assessed by RT-PCR
analysis of the $F3'5'H$ transcript. Sequencing of the obtained cDNA product revealed the
presence of a MITE belonging to *Stowaway*, named $dTstu1$. This element was absent
from the $F3'5'H$ transcript in ‘JKP’, which was analyzed in parallel (Figure 2). In support
of this, Southern blot analysis with $F3'5'H$ cDNA from ‘JKP’ as a probe demonstrated a
reduction in size in ‘JKP’ of a 5kb $Eco$RV fragment present in ‘72218’ and ‘Jaga kids red’
(‘JKR’), which is somaclonal cultivar with red tubers simultaneously obtained from the
leaf protoplast culture of ‘72218’ that yielded ‘JKP’ (OKAMURA 1991, 1994). Genomic
sequence analysis of $F3'5'H$ genes from ‘72218’ and ‘JKP’ revealed that the only
difference between the full-length genes is the insertion of $dTstu1$ into the first exon of
$F3'5'H$ in ‘72218’ (designated $f3'5'h::dTstu1$, DDBJ accession AB496977). This element
was not present in $F3'5'H$ of ‘JKP’ (named $F3'5'H::rev$, DDBJ accession AB496976),
which explained the size difference observed in Southern blot analysis (Figure 3A and B).
As the result of a stop codon within $dTstu1$, $f3'5'h::dTstu1$ should produce a truncated
protein of only 24 amino acid residues in ‘72218’, whereas $F3'5'H::rev$ codes for a
functional full length protein of 510 amino acid residues, one residue longer than
predicted for the wild type which was reported as a functional $F3'5'H$ gene of diploid
potato clone W5281.2 (GenBank accession AY675558, JUNG et al. 2005).

At most, three copies of F3’5’H were deduced to exist in ‘72218’ and ‘JKP’ based on the results of Southern blot and genomic sequence analyses. Apart from the full-length F3’5’H, the triploid ‘72218’ and ‘JKP’ possess two truncated copies of this gene (f3’5’h2 and f3’5’h3, DDBJ accession AB496978 and AB496979) (Figure 3B). The sequences of each pseudogene were completely identical between ‘72218’ and ‘JKP’. Both f3’5’h::dTstu1 and F3’5’H::rev have an EcoRV recognition site at the middle of the gene, which is absent in 7.8 kb of determined f3’5’h2 sequence. Therefore, the largest band in Figure 3A represents f3’5’h2, while the 6.3 kb fragment is derived from the third allele, f3’5’h3, which only contains the latter half of the third exon, encoding the P450 signature motif conserved among all known plant F3’5’H genes. This motif is lacking in f3’5’h2, which strongly suggests that transcripts of this copy do not function properly. Triploid red ‘72218’ has only pseudo copies of the gene, f3’5’h::dTstu1, f3’5’h2 and f3’5’h3. Its purple somaclonal variant, ‘JKP’, has three copies of the gene F3’5’h::rev, f3’5’h2 and f3’5’h3.

As F3’5’H::rev is the only allele able to produce a full-length, non-defective protein, we conclude that excision of dTstu1 from f3’5’h::dTstu1 during the establishment of ‘JKP’ is the major reason for the color change from red to purple.

An active Stowaway MITE, dTstu1: The sequence of dTstu1 is short (239 bp), A/T rich (67%) and marked by TIRs corresponding to the consensus CTCCCTCYGTC and a duplication of the TA target sequence at the insertion site, all characteristics of Stowaway
MITEs (BUREAU and WESSLER 1994). The formation of DNA secondary structure is predicted for this element as well (Figure 3C). Database searches retrieved sequences similar to \(dTstu1\) not only in genomes of \(Solanum\) but also in the other \(Solanaceae\) plants, for example, \(Capsicum, Petunia\) or \(Nicotiana\) (GenBank accession DQ309518, AY136628, AF277455).

Comparison of the wild type \(F3'5'H\) gene with that of ‘JKP’ confirmed the addition of one amino acid residue (valine) generated by a three nucleotide insertion, GTA, in \(F3'5'H::rev\) (Figure 3C). These nucleotides could be traced to consist of one base (G) derived from \(dTstu1\) and two (TA) from the duplicated target site. This duplication was also present in the disrupted \(f3'5'h::dTstu1\) of ‘72218’ and leading to the observed size difference of 238 bp between the transcripts derived from these genes. Therefore, the presence of these three nucleotides in \(F3'5'H::rev\) of ‘JKP’ strongly supports that the 239 bp \(dTstu1\) was excised from \(f3'5'h::dTstu1\) in ‘72218’ as a transposable element leaving a footprint which is normally associated with transposase-mediated excision. We conclude that the \(F3'5'H\) gene in ‘72218’ (red) had become functionless as a result of \(dTstu1\) insertion and then reverted in ‘JKP’ (purple), presumably by transposition of \(dTstu1\) during culturing.

Another active \(dTstu1\)-like Stowaway MITE, \(dTstu1-2\): Excision of \(dTstu1\) from the \(F3'5'H\) gene during culturing of leaf protoplasts derived from ‘72218’, raised the possibility that other \(dTstu1\)-like Stowaway MITEs had undergone transposition under these conditions. In support of this, we isolated an extra \(dTstu1\)-like element specific for
‘JKP’ by use of a DNA-fingerprinting technique adapted from a method with which inter-MITE polymorphisms were detected. With this method, multiple regions between MITEs had been amplified by PCR using a primer annealing to TIRs in the outer direction (CHANG et al. 2001). By using primers specific for the \(dTstu1\) internal sequence (instead of the TIRs sequences), we obtained a product for ‘JKP’ not observed for ‘72218’ which contained an element almost identical to \(dTstu1\), named \(dTstu1-2\) (DDBJ accession AB496980). After identification of the flanking regions, PCR amplification of the region containing the site of integration of \(dTstu1-2\) in ‘JKP’ produced in ‘72218’ a fragment of one size, not containing the transposable element. In ‘JKP’, however, two fragments, one with and the other without \(dTstu1-2\), were detected (Figure 4A), suggesting that no alleles of the locus carried the transposable element in ‘72218’ and that \(dTstu1-2\) had been newly inserted in an allele. Comparison of the sequence surrounding the insertion site confirmed the presence of a duplicated TA di-nucleotide, which is the target sequence of Stowaway MITEs (Figure 4B). Compared to \(dTstu1\), \(dTstu1-2\) had a similar length, 239 bp, but contained four base changes, two of which were in the TIRs (Figure 5). These changes made the TIRs of \(dTstu1-2\) more complementary to each other than in the case of \(dTstu1\). Therefore, in view of a comparable propensity for transposition, this Stowaway MITE conceivably was mobilized under the same conditions that caused \(dTstu1\) to be excised from the \(F3'5'H\) gene. If this is the case, activation of transposition of these MITEs was induced by culturing.
In order to survey the active MITE copies related to \textit{dTstu1}, we carried out MITE display using primers designed from the sequences of \textit{dTstu1} and \textit{dTstu1-2}. More than fifty peaks were detected but slight differences existed among ‘72218’, ‘JKR’ and ‘JKP’. ‘JKR’ revealed three new peaks and ‘JKP’ exhibited three new peaks and a missing peak as compared with ‘72218’ when using a primer with selective nucleotide T (Figure S2). The insertion of \textit{dTstu1-2} in ‘JKP’ was visualized as a new peak at the expected position of 315 bases in size but the excision of \textit{dTstu1} in ‘JKP’ was not detected at the expected position of 50 bases due to the signal of the other putative insertion at the same position. Although most of the peaks were identical a few polymorphisms were detected among the three clones.

\textbf{DISCUSSION}

In this study we found the first active \textit{Stowaway} MITEs in dicotyledons and presented the evidence of their movement. Excision of \textit{dTstu1} caused a somaclonal variation of skin color in potato tubers. Insertion of \textit{dTstu1-2} was observed at another locus in the genome of the same somaclonal variant, ‘JKP’. It became obvious that two major groups of MITEs, \textit{Stowaway} and \textit{Tourist}, have the potential to transpose in plants. Movement of MITEs had not been proven for a long time because most of them are not inserted into genes (OKI \textit{et al.} 2008) with the possibility to cause an altered phenotype and because the high copy number of MITE in the genome precludes analysis of their individual movement. 'Fingerprints' of MITE abundance, obtained by Southern hybridization with
MITE DNA probes (NAITO et al. 2006), showed differences among strains, which suggested movement of MITEs but did not provide direct evidence for their transposition. Previously, a case in which MITE transposition resulted in a phenotypic change has been reported. A MITE named *mPing*, belonging to *Tourist*, was found to be inserted in the rice *Rurm1* gene causing the slender glume phenotype which reverted to wild type by excision of the mobile element (NAKAZAKI et al. 2003). We present in this report another rare case of a MITE giving rise to an altered phenotype, namely that of *dTstu1* belonging to *Stowaway*. We found this MITE to disrupt the *F3’5’H* gene of a potato clone (‘72218’) resulting in a red tuber color. Due to the excision of *dTstu1* tuber color changed to purple in the somaclonal variant. Thus, in two cases, visible phenotypes, the grain shape for *mPing* and the tuber color for *dTstu1*, provided strong evidence for the movement of MITEs belonging to *Tourist* and *Stowaway* respectively.

As described in this report, the duplication of the target sequence TA at the insertion site of *dTstu1* was observed for the *F3’5’H* gene of ‘72218’. The footprint left behind in *F3’5’H::rev* in ‘JKP’ suggests that the excision is catalyzed by a transposase. By lack of any open reading frame, the short *Stowaway* MITEs of both *dTstu1* and *dTstu1-2* are not able to code for such a transposase, which has to originate from other, unrelated transposable elements as found in the case of *mPing*. This *Tourist* MITE was mobilized by transposases derived from the *Ping* and *Pong* transposable elements (YANG et al. 2007). Mobile *dTstu1* and *dTstu1-2* enable us to search for transposases that control *Stowaway* MITEs. The *Mariner*-like element (MLE) is one of the most widely distributed
transposable elements in eukaryotes and its transposase can interact in vitro with TIRs of a *Stowaway* MITE (FESCHOTTE *et al.* 2005). Using yeast cells, MLE transposases of rice were proved to actually activated transposition of *Stowaway* MITEs of rice. (YANG *et al.* 2009). MLE is a good candidate for a source of transposase for *dTstu1* movement.

Our results show that the activation of *Stowaway* MITEs not only involves a transposase but appears to occur under particular conditions. MITE displays of regenerated plants from protoplasts indicated that most of the MITE insertion sites were maintained, although a few differences emerged during tissue culture. The observed differences in sequences and in the insertion sites between the silent copies and the active ones should be investigated further as these may reveal the factors for transposition. Tissue culturing causes the activation of various transposable elements (PESCHKE *et al.* 1987; GRANDBASTEIN *et al.* 1989; PESCHKE and PHILLIPS 1991; HIROCHIKA 1993; JIANG *et al.* 2003; KIKUCHI *et al.* 2003). It was observed that the conditions under which *dTstu1* (and possibly *dTstu1-2*) was excised, i.e. at some time during the culturing of leaf protoplasts isolated from ‘72218’, caused 7% of the regenerated plants to bear purple tubers instead of the parental red potatoes (OKAMURA 1991).

Furthermore, red tubers with small purple sectors were found in some regenerated plants that originated from cultured leaf protoplasts of ‘72218’ (Figure S3). Such chimeric tubers or purple tubers, however, have not been found in tuber-propagated ‘72218’ plants, which are clonally reproduced as seed potatoes in the field. These facts also support the importance of cell culture conditions for the activation of *dTstu1*. It remains to be seen
how tissue culturing confers the activation. Alteration of the epigenetic status by DNA de-methylation of the element itself or of the genes encoding its transposase has been reported to activate a transposable element during tissue culture (KAEPPLER et al. 2000; CHENG et al. 2006; LISCH 2009) and could therefore be part of the reason.

How MITEs have spread over various genomes and in such high numbers is still obscure but poses one of the important questions to be tackled in order to comprehend the evolution of the eukaryotic genome. Active MITEs, like \textit{dTstu1}, can provide a tool for this investigation.

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LITERATURE CITED


BUREAU, T. E. and S. R. WESSLER, 1992 \textit{Tourist}: A large family of small inverted


TOGURI, T., M. AZUMA and T. OHTANI, 1993 The cloning and characterization of a cDNA encoding a cytochrome P450 from the flowers of *Petunia hybrida*. Plant Sci. **94**: 119-126.

TOGURI, T., N. UMEMOTO, O. KOBAYASHI and T. OHTANI, 1993 Activation of anthocyanin synthesis genes by white light in eggplant hypocotyl tissues, and identification of an inducible P-450 cDNA. Plant Mol. Biol. **23**: 933-946.


FIGURE 1.-Tuber pigmentation of ‘72218’ and ‘Jaga kids purple’ (‘JKP’). (A) Tuber appearance of ‘72218’ and ‘JKP’. (B) Schematic pathway of anthocyanidin biosynthesis. Enzyme abbreviations are as follows: \( F3H \), flavanone 3-hydroxylase; \( F3'5'H \), flavonoid 3',5'- hydroxylase; \( DFR \), dihydroflavonol 4-reductase; \( ANS \), anthocyanidin synthase; \( MT \), anthocyanin 3'-methyltransferase.
FIGURE 2.- Flavonoid 3',5'-hydroxylase (F3'5'H) transcripts in '72218', 'JKR' and 'JKP'. (A) RT-PCR products specific for F3'5'H gene using cDNAs synthesized from RNAs prepared from tuber skins of '72218', 'JKR' and 'JKP' as templates. The migration of molecular weight markers are shown on the right. (B) Schematic structure of cDNAs for F3'5'H gene in '72218', 'JKR' (1) and 'JKP' (2). Shaded boxes indicate the coding regions of F3'5'H genes, thin lines the non-coding regions. The black box depicts the insertion of dTstu1.
FIGURE 3.- Flavonoid 3',5'-hydroxylase (F3'5'H) genes in '72218' and 'JKP'. (A) Southern blot analysis of genomic DNA digested with EcoRV and probed with a labeled RT-PCR product of F3'5'H::rev. Approximate sizes are given on the left. The largest band represents f3'5'h2 since the EcoRV recognition site is absent in 7.8 kb of determined sequence. The 6.3 kb fragment is derived from f3'5'h3. The rest of the bands represent f3'5'h::dTstu1 or F3'5'H::rev since both f3'5'h::dTstu1 and F3'5'H::rev have an EcoRV recognition site at the middle of the gene. (B) Structure comparison of F3'5'H genes. Both f3'5'h2 and f3'5'h3 are incomplete genes, f3'5'h2 lacks the latter half of the third exon and f3'5'h3 contains only the latter half of the third exon. Triploid red '72218' has only pseudo genes, f3'5'h::dTstu1, f3'5'h2 and f3'5'h3. Triploid purple 'JKP', somaclonal variant of '72218', has F3'5'H::rev, f3'5'h2 and f3'5'h3. Coding regions (shaded boxes) are separated by introns (lines) with the dTstu1 insertion depicted by a black bar. Arrows indicate the EcoRV recognition site in f3'5'H::dTstu1 and F3'5'H::rev. (C) Structure of dTstu1 and the nucleotide and amino acid sequences of F3'5'H genes proximal to the dTstu1 insertion site. Wild type is the previously reported functional F3'5'H gene (JUNG et al. 2005). A pair of vertical sequences shows the TIRs where complementary sequences are hyphenated. An asterisk indicates a stop codon present in f3'5'h::dTstu1. The footprint remaining after dTstu1 excision (including the duplicated TA target site) is underlined.
Figure 4.- Insertion of \textit{dTstu1-2} in 'JKP' occurring as a somaclonal variation. (A) PCR-amplified genomic region proximal to the \textit{dTstu1-2} target site in 'JKP' in comparison with '72218'. Insertion of \textit{dTstu1-2} yielded the larger amplified fragment in 'JKP'. The migration of molecular weight markers is shown on the right. (B) Nucleotide sequences around the \textit{dTstu1-2} insertion site in 'JKP' (1) and '72218' (2). The pair of vertical sequences represents the TIRs where hyphens connect complementary nucleotides. The target sequence TA and its duplication are underlined.
FIGURE 5.- Sequence comparison between $dTstu1$ and $dTstu1-2$. Arrows indicate the sequences of TIRs. Nucleotide differences are marked by asterisks.