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A LINE-type retrotransposon active in meristem stem cells causes heritable transpositions in the sweet potato genome

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Abstract

We isolated a LINE-type retrotransposon, *LIb*, which showed high transposition activity in sweet potato callus. A copy transposed in the callus was 6303 bp in length and showed key features of a LINE element. Apparently full-length copies sharing the 5′ UTR sequence with the 6303-bp copy increased dramatically in the callus as several original copies in the sweet potato genome. These apparently full-length copies had almost identical sequences to other transposed copies, many of which were truncated at the 5′ end upon transposition. These results indicate that active *LIb* is confined to a single LINE family, and that members containing a long functional 5′ UTR are present in limited numbers in the sweet potato genome. This is despite their copy numbers being estimated at over 100. The transcription of *LIb* was not completely suppressed, even in wild-type plants. Spontaneous transpositions were found among local variant lines of the cultivar Koukei14, from which the callus with high *LIb* activity was derived. Meristem culture of this cultivar appeared to facilitate transpositions of *LIb* in a mericlone plant. This is the first experimental demonstration of retrotransposition in a plant species without the imposition of cell differentiation. *LIb* transpositions appear to occur in single founder cells in the meristem because the *LIb* insertion was found throughout mericlone plant tissues. Transpositional activities in meristem cells might be essential characteristics of plant retrotransposons that cause heritable changes in host plant genomes and genetic systems.

Abbreviations: LINE, long interspersed nucleotide element; LTR, long terminal repeat; MCS, multiple cloning site; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RTase, reverse transcriptase; RT-PCR, reverse transcriptase-polymerase chain reaction; SINE, short interspersed nucleotide element; S-SAP, sequence-specific amplification polymorphism; TPRT, target-primed reverse transcription; TSD, target site duplication; UTR, untranslated region

Introduction

Retrotransposons are mobile genetic elements that require the reverse transcription of an RNA intermediate to move through the genome (Kumar and Bennetzen, 1999). They are classified into two categories depending on whether they possess long terminal repeats (LTRs). Non-LTR retrotransposons are sub-classified into long interspersed nucleotide elements (LINEs) and short interspersed nucleotide elements (SINEs). Full-length LINEs are usually several kilobase pairs (kbp) in length and have two open reading frames (ORFs) that encode endonuclease, reverse transcriptase (RTase), and other proteins required for retrotransposition (Mathias *et al.*, 1991; Feng *et al.*, 1996). SINEs are normally 80–400 bp long and do not encode proteins (Ohshima *et al.*, 1996). It is postulated that the proteins encoded by a LINE function in *trans* to mobilize SINEs (Wei *et al.*, 2001).

Non-LTR retrotransposons are found at high copy numbers in mammals and modify genomic organization and function (Kazazian, 2000). However, most LINEs in the mammal genome are defective in transposition. Although LINE-1 (L1) retrotransposons are present in over 500 000 copies and comprise 17% of the human genome, only 50–100 L1s are competent in the average human (Sassaman *et al*., 1997; Park and Kazazian, 2000; Brouha *et al*., 2003). Nevertheless, *de novo* L1 transposition in germ lines or somatic cells accounts for a number of genetic disorders or polymorphisms among human individuals (Ostertag and Kazazian, 2001).

LINE-type elements are also found in plant species, although LTR retrotransposons appear to be most prevalent in plant genomes. *Cin4* is the first plant L1-like element discovered in *Zea mays* (Schwarz-Sommer *et al*., 1987). Other closely related elements are found in the genomes of *Arabidopsis thaliana* (*Tal1* and *ATLN*), *Lilium speciosum* (*del2*), and a wide variety of plant species (Leeton and Smyth, 1993; Wright *et al*., 1996; Ohta *et al*., 2002). Recently, the first active plant LINE-type retrotransposon was reported in rice (Komatsu *et al*., 2003). Because non-LTR retrotransposons are ubiquitous among plants, active elements should have exerted a significant impact on plant genome evolution and they may still be affecting plant genetic systems as mutagens. Virtually nothing is known about the mechanisms and regulation of non-LTR retrotransposons in plants, so the identification of an active LINE-type element is crucial.

We recently isolated an active LTR retrotransposon, *Rtsp-1*, from the sweet potato (*Ipomoea batatas* [L.] Lam.) genome, which was transposed in callus tissue (Tahara *et al*., 2004). When further examining the same callus line, we found that a non-LTR retrotransposon element was also transcribed in the callus. In this paper, we describe the identification of an active LINE-type retrotransposon in the sweet potato genome that was actively transposed in callus during tissue culture. We named this retrotransposon *LIb* (LINE-type retrotransposon in *I. batatas*). *LIb* transposed spontaneously in the cultivar from which it was isolated. Furthermore, shoot meristem culture appeared to facilitate its transposition. Interestingly, evidence indicates that *LIb* transposition occurred in meristematic stem cells and has introduced inherited changes into the sweet potato genome.

Materials and methods

Plant material

The sweet potato cultivar used in this study was Koukei14. This cultivar originated from a single F_1 plant and was vegetatively propagated. It has been cultivated in a wide area in the western part of Japan since its release in 1945 and a number of local lines have diverged. The lines analyzed in this study are listed in Table 1. Plants of these lines were grown in a field during the warm seasons and maintained in a greenhouse over winter. Koukei14 apices were grown in meristem culture, as described previously (Tahara *et al*., 2004).

Callus line

Callus line KB-4 was initiated from the callus formed at the basal part of the shoot apex, which

Table 1. Local lines diverged from a sweet potato cultivar, Koukei14, used in the study.

JP number[a]	Cultivar or line name	Description
168607	Koukei14	Original collection from the breeding station
–	Koukei14 (SZ)	Line used in Okayama University
91320	Koukei14 (Chugoku)	Recurrent collection from the breeding station
91321	Koukei14 (Oami-Shirasato)	Collection from a grower in Chiba Prefecture
–	Tosabeni	Local line in Kouchi Prefecture
179006	Naruto-kintoki	Local line in Tokushima Prefecture
–	Naruto-kintoki CL3	Elite clone of the Growers' Association at Matsushige in Tokushima Prefecture
–	Naruto-kintoki CL6-1	Elite clone of the Growers' Association at Matsushige in Tokushima Prefecture
–	Naruto-kintoki CL6-2	Root discoloration mutant from CL6-1
–	Naruto-kintoki CL6-3	Root discoloration mutant from CL6-1

[a] JP number is the accession number for a sweet potato germplasm collection by National Institute of Crop Science in Japan.

showed abnormal differentiation during meristem culture of Koukei14 (SZ) (Tahara *et al*., 2004). The abnormal root tissue of KB-4 was harvested and cultured on the same MS medium; KB-4R is the callus formed in this root culture. The callus lines were maintained by transferring them onto fresh medium at regular intervals.

Nucleic acid extraction

Genomic DNA and RNA were extracted from plant materials using DNeasy and RNeasy Plant Mini Kits, respectively, according to the manufacturer's recommendations (Qiagen, Hilden, Germany). The RNA was tested by β-actin gene amplification to ensure the absence of detectable DNA contamination, as described previously (Tahara *et al*., 2004).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed on cDNAs prepared from total RNAs of the leaves and roots of wild-type field-grown Koukei14 (SZ) plants, KB-4 and KB-4R calluses, the leaves of mericlone plantlets of Koukei14 (SZ), and callus formed at the basal part of the plantlet. The primer pair used in the analysis was designed to amplify a 362-bp fragment at the 3′ end of ORF2: RT-D, 5′-AACA GTTTCCTAGCCGAGTTATGG-3′ and RT-A, 5′-AGTTATCGCCGTCTGCTAGAGG-3′ (Figure 3).

Sequence-specific amplification polymorphism (S-SAP)

S-SAP was performed using the procedure described previously (Tahara *et al*., 2004), except that (1) the LTR retrotransposon primers were replaced with the *LIb*-sequence-specific primers, L-D4, 5′-ACATTCTTCGCGAAGGCAACCAA T-3′, and L-D3, 5′-CCGATGATCTGAGGATT TTTCTCC-3′ (Figure 3); (2) *Alu*I was used to digest the genomic DNA in addition to *Mse*I; and (3) the *Mse*I adapter was replaced with an adapter developed for suppression PCR (Siebert *et al*., 1995). The L-D3 primer was labeled with Texas Red at the 5′ end. Conditions for DNA digestion and adapter ligation used for *Alu*I were the same as those used for *Mse*I in our previous study (Tahara *et al*., 2004). The suppression PCR adapter was used to effectively suppress PCR amplification of non-specific adapter-ligated fragments. *Alu*I leaves a blunt-digested end. The adapter 5′-CTAATACGACTCACTATAGGGC TCGAGCGGCCGCCCGGGCAGGT-3′ was annealed to 5′-ACCTGCCC-NH_2-3′. For the *Mse*I adapter, the latter oligonucleotide was replaced with 5′-TAACCTGCCC-NH_2-3′ to form the *Mse*I sticky end. To selectively amplify and label fragments containing the 3′ end of *LIb* and the adjacent host sequence, two rounds of PCR were run with nested primers. For the *Mse*I preparation, the first round of PCR contained 2 μl of the ligation mixture as template in a total reaction mixture of 10 μl, and the L-D4 and AP1 primers, where AP1 is the adapter primer 5′-GGATCCTAATACGACTCACTATAGGGC-3′. The second PCR contained 1 μl of a 40-fold dilution of the first PCR product in a 10 μl reaction mixture, and the labeled L-D3 and AP2 primers, where AP2 is the nested adapter primer, contained 5′-AATAGGGCTCGAGCGGC-3′. For the *Alu*I preparation, the first-round PCR contained 2 μl of a 5-fold dilution of the ligation mixture as template in a 10 μl reaction mixture, and the second PCR contained 1 μl of a 100-fold dilution of the first PCR product in a 10 μl reaction mixture. Primer combinations were the same as for the *Mse*I preparation. Apart from the template and primers, the PCR reaction mixture was as described previously (Tahara *et al*., 2004). The cycling profile was 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min. A final extension at 72 °C for 2 min was included for all S-SAP PCR reactions. Electrophoresis was performed and S-SAP products of interest were cloned and sequenced following the procedures described previously (Tahara *et al*., 2004).

Cloning the entire sequences of transposed LIb *copies at new insertion sites*

The insertion site of the newly transposed *LIb* copy was cloned from a polymorphic S-SAP product as the fragment at the 3′ end of the *LIb* copy ligated to the host sweet potato genome. Suppression PCR was carried out based on the host sequence of the S-SAP product to amplify the interrupted host sequence flanking the 5′ end of the *LIb* insert, with the homologous chromosome without the insert in Koukei14, Koukei14 (SZ) or

Naruto-kintoki plants used as template. The primer was designed based on the flanking sequence, and the entire transposed *LIb* copy was amplified together with adjacent host fragments. The sequence was determined for an apparently full-length copy of *LIb* that had transposed during tissue culture. Then another set of suppression PCRs was performed based on the 5′ sequence of the transposed copy to obtain other full-length copies of *LIb* transposed into different genomic sites during tissue culture. These suppression PCRs facilitated the cloning of various insertion sites with the 5′ sequences of the transposed *LIb* copies. Other suppression PCRs were conducted to obtain the host sequences flanking the 3′ ends of the copies at these insertion sites. After sequencing, a primer was designed and the fragment containing the entire *LIb* insert was amplified. The pair of primers designed for each suppression PCR is shown in Table 2. The primer combination for the first round of suppression PCR was A1/AP1 at each site or *LIb*-S14-A1/AP1, and that for the second nested PCR was A2/AP2 at each site or *LIb*-S14-A2/AP2. DNA purified from Koukei14 leaves (2 μg) was digested with 10 units of *Alu*I, *Ssp*I, or *Sca*I using the reaction buffer supplied by the manufacturer (New England Biolabs, Ipswich, MA). This was ligated to the

Table 2. Primers used for suppression PCR and PCR amplification of the *LIb* insert.

Site or sequence	Primer	Primer sequence (5′-3′)	Sequence used to design primers/amplification target
S14	A1	GTGCAAACCGCTTTTTTGCCCAAA	KB-4 genome sequence/interrupted host sequence adjacent the 5′ end of *LIb*
	A2	CCACTGCACAACTGATATTGCCAT	
	D1	ATCTTGAGAGTTGCAGACTAGCAG	
LIb at S14	*LIb*-S14-A1	AACCAGAACTGCAGAAGCCGGAGGT	Transposed *LIb* copy at S14/other new insertion sites in KB-4
	LIb-S14-A2	GAAAACCTAGCGGAGCCCTGTTACT	
S10	A1	CTCAAATCCTACCGTCCACGTCAT	KB-4 genome sequence/interrupted host sequence adjacent the 3′ end of *LIb*
	A2	ACCGTCCACGTCATACATATAAATCAC	
	D1	GAGAGTGCGGATGATCTATTGAAGC	
S11	A1	CTGGCAAAAACTTGAGTCAGATTGTCTC	KB-4 genome sequence/interrupted host sequence adjacent the 3′ end of *LIb*
	A2	CAGGTCTTAATCTGTGAGACAGGC	
	D1	CGAGCCACTGCATACCCAACAATTA	
S12	A1	CTGATTATGCTGGTCATCCTAATTAC	KB-4 genome sequence/interrupted host sequence adjacent the 3′ end of *LIb*
	A2	GTTCTGTTTGTGATGTCTCTCAGGGA	
	D1	CCCAAACTGATAAAGAACTGAAAGAGAATCC	
S25	A1	TGCCTAGGCAGTCAACATATCTCTAG	KB-4 genome sequence/interrupted host sequence adjacent the 3′ end of *LIb*
	A2	CTAGCATATTCCACCTAGATCTCATTCAAC	
	D1	CCCATCGTTAGCATCAAGCTTGTAGA	
S34	A1	GCATGGAGACAGAAATGGTAGACG	KB-4 genome sequence/interrupted host sequence adjacent the 3′ end of *LIb*
	A2	CAGGAAAACGACTGATGAAGTGGC	
	D1	GTCGAGTAACTAACCCGAGTCACA	
SCL3	A1	AAGTTGGATCCTAAAATAGTGGCAAGAGAT	Naruto-kintoki CL3 genome sequence/interrupted host sequence adjacent the 5′ end of *LIb*
	A2	CATCAAGCCACTAGGACAATTACC	
	D1	CACGAATTATTGGTTAGTTGACTGACCTCA	
SCL6	A1	ATGATTGGGGTGTAGTCTTTACCC	Naruto-kintoki CL6-1 genome sequence/interrupted host sequence adjacent the 5′ end of *LIb*
	A2	ACCTCTCGTTTGCTAGAAAAGGTG	
	D1	CTTCTTGTGAGGGTGACTCACTTA	
SKO	A1	CTTTCAGTTAGCTCCCTCTAATCTCTC	Koukei14 (SZ) genome sequence/interrupted host sequence adjacent the 5′ end of *LIb*
	A2	CCATACTTTTTCATTCCTGCCAAGCCA	
	D1	GTGTGGACTGCCTTACCTTGTGTCAAG	
SNA	A1	CGGCAATAAAGGAGTTGATGGGAC	Naruto-kintoki genome sequence/interrupted host sequence adjacent the 5′ end of *LIb*
	A2	CATGGGAATGTGTGAAGACCTTGAGG	
	D1	CATCTCCTGCACATTCTCCGTCATC	
SY30	A1	TCATGGTAAGGTCTTGTAGGGG	Mericlone plantlet 4 genome sequence/interrupted host sequence adjacent the 5′ end of *LIb*
	A2	AGGGACAACTCCTCAATAGGATC	
	D1	CAGCGACCAATCGAATATGCTACC	

The primers of A1 and A2 were designed for suppression PCR, whereas the D1 primer was used with either A1 or A2 primer in PCR to amplify the fragment containing an *LIb* insert.

aforementioned *Alu*I adapter because these restriction enzymes produce a blunt DNA end after digestion.

For each restriction enzyme preparation, the first-round PCR contained 1 μl of a 5-fold dilution of the ligation mixture as template in a 10 μl reaction mixture, and the second PCR contained 1 μl of a 100-fold dilution of the first PCR product in a 10 μl reaction. *Ex Taq* (TaKaRa, Tokyo, Japan) was used according to the manufacturer's instructions. The cycling profile was 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 3 min. A final extension at 72 °C for 5 min was included for all suppression PCR reactions.

PCR to amplify the entire *LIb* copies at newly transposed sites was performed as suppression PCR. Exceptions were that the D1 primer was used in combination with either the A1 or A2 primer at each site (Table 2), and the extension time during the PCR cycle was 6 min.

The TOPO TA or XL Cloning Kit (Invitrogen, Carlsbad, CA) was used to clone PCR products depending on their length. DNA sequencing followed the method described in our previous study (Tahara *et al.*, 2004).

Southern blotting analysis

A pair of DNA fragments was prepared from the pCR-XL-TOPO plasmid containing the *LIb* copy transposed at the S14 site of the KB-4 callus. The 5′ probe was a *Sac*I-digested plasmid containing an entire 5′ UTR (566 bp) and the 5′ portion of ORF1 (587 bp) together with the adjacent genome sequence and a plasmid multiple-cloning site (MCS) of 160 bp. The 3′ probe was a *Pvu*I- and *Pst*I-digested plasmid containing the 3′ portion of ORF2 (999 bp), the 3′ UTR (30 bp), and the poly-adenylated tail (11 bp) together with the adjacent genome sequence and a plasmid MCS of 52 bp (shown diagrammatically in Figure 4).

After restriction enzyme digestion, these fragments were purified by agarose gel electrophoresis and labeled with digoxigenin by random priming with the DIG-High Prime DNA Labeling and Detection Kit (Roche Diagnostics, Tokyo, Japan). Genomic DNA (10–16 μg) from Koukei14 (SZ) and Naruto-kintoki CL3 plants, as well as from KB-4 and KB-4R calluses, was digested with *Ase*I for 20 h. The fragments were purified by phenol/chloroform extraction and separated electrophoretically on 1% agarose gel before being transferred onto Hybond N+ membranes (Amersham Biosciences, Piscataway, NJ). Hybridization was carried out according to the manual for the DIG kit with a high-stringency wash of 0.1×SSC and 0.1% SDS at 68 °C for 20 min. A fluorographic image was detected with a Fluor-S MAX Multi-Imager (Bio-Rad Laboratories Hercule, CA).

Dot-blot analysis

Dot-blot hybridization was used to determine the number of apparently full-length *LIb* copies and all detectable copies, including 5′ truncated ones, in the sweet potato genome using 5′ and 3′ Southern probes, respectively. Serial dilutions of the *LIb*-containing plasmid and genomic DNA from Koukei14 (SZ) were spotted onto Hybond N+ membranes as three replicates. The plasmid standards contained 0.01, 0.05, 0.1, 0.5, and 1 ng of DNA, whereas genomic DNA dots contained 250, 500, and 750 ng of DNA. DNA was quantified on agarose gels stained with ethidium bromide. Hybridization and signal detection were performed as in the Southern analysis. Within the range of 0.01–0.1 ng of standard DNA, a robust linear equation was obtained between hybridization signals and plasmid DNA quantity. The relative amount of *LIb* DNA present in an amount of spotted genomic DNA was interpolated, and the copy number of *LIb* was calculated assuming the sweet potato genome was 1597 Mb/1C, where 1C is the haploid genome (Arumuganathan and Earle, 1991).

Results

Transcription and transposition of LIb *in cultured cells*

Transcription of *LIb* was unexpectedly discovered during the 3′ rapid amplification of cDNA ends (RACE) analysis of KB-4 callus tissue. The oligo-dT primers used in the 3′ RACE reaction amplified a cDNA fragment between the thymidine-rich sequence in ORF2 and the poly-adenylated tail of *LIb*, as in the aligned sequences in Figure 3. A Blast search of the DNA database revealed significant homology with the reverse transcriptase of LINEs.

RT-PCR with the cloned *LIb* sequence confirmed transcription not only in KB-4 and KB-4R calluses, but also in the leaves and roots of wild-type field-grown Koukei14 (SZ) plants. However, transcript accumulation was relatively low in the leaves and roots (Figure 1). *LIb* was similarly transcribed in the young leaves of mericlone plantlets and calluses formed during the meristem culture of Koukei14.

S-SAP based on the 3′ sequence of *LIb* revealed a large increase in the number of S-SAP products from the callus lines (Figure 2). These results indicate that *LIb* was transposed in callus cells during tissue culture at substantial frequencies.

Transposed copies in cultured cells

S-SAP products from KB-4 callus were cloned and four were sequenced. Each fragment contained the 3′ terminal sequence of *LIb* at one end and a flanking host sequence at the other. PCR to amplify the fragment between the *LIb* insert and flanking host sequence at each insertion site was performed for KB-4 and Koukei14 (SZ), from which KB-4 was derived. PCR products of the expected size were observed at three sites for KB-4 only, whereas a product was observed for both KB-4 and Koukei14 (SZ) at one site (data not shown). The junctions between *LIb* and the host sequence at these three sites were most likely generated by transpositional insertion of *LIb* during tissue culture of KB-4. One of these

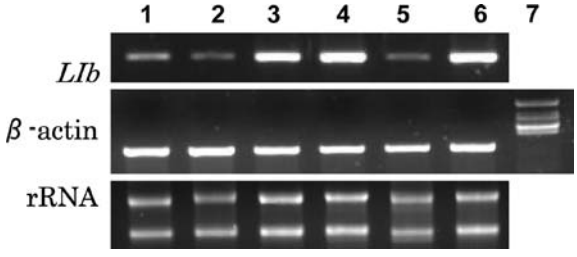

Figure 1. RT-PCR analysis of the LINE-type retrotransposon of sweet potato, *LIb*. Top row: Fragments amplified with primers corresponding to the 3′ end of ORF2 of *LIb* using as templates cDNAs from leaves (lane 1) and roots (lane 2) of wild-type Koukei14 (SZ) plants, KB-4 (lane 3), and KB-4R (lane 4) of Koukei14-derived callus lines, leaves of mericlone plantlets (lane 5), and calluses formed at the basal part of the plantlet (lane 6). Middle row: Fragments amplified with primers between two exons of the *β*-actin gene. Genomic DNA from a Koukei14 plant (lane 7) was included as a control to ensure the absence of detectable DNA contamination in cDNA samples. Bottom row: rRNAs in the extracted RNA samples used for cDNA synthesis.

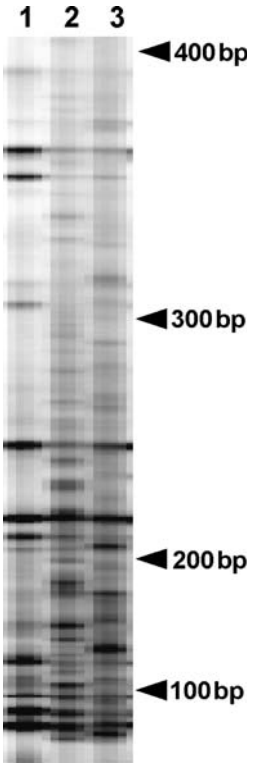

Figure 2. Sequence-specific amplification polymorphism (S-SAP) fluorographs from the 3′ sequence of *LIb* in Koukei14 (SZ) plants (lane 1) and the Koukei14-derived callus lines, KB-4 (lane 2) and KB-4R (lane 3), using *Alu*I as the restriction enzyme. Throughout the S-SAP fluorographs in this paper, fragment sizes are indicated based on sequencing reaction mixtures of a DNA fragment of known sequence separated electrophoretically adjacent to samples.

Figure 3. Aligned sequences of the 3′ RACE product, the cloned copy of transposed *LIb* (*LIb* at 14), and S-SAP and suppression PCR (Site without insert) products at the S14 site. For clarity, the inner portions of 720 and 5195 bp are omitted from the 'Site without insert' and *LIb* sequences, respectively. Adapter sequences are not included in the sequences of the 'Site without insert' and 'S-SAP product'. The D1 primer was used in PCR with the A2 primer to clone an entire transposed *LIb* copy at this site from the KB callus. The duplicated target-site sequences (target-site duplications) are boxed. Bold underlined letters correspond to the PCR primers. Asterisks (*) indicate sequence identity to either the 'Site without insert' sequence or the transposed *LIb* sequence (*LIb* at 14). Nucleotides that differ from the reference sequences are shown in capital letters. Dashes indicate absence of nucleotides at the corresponding positions. The sequences of the 3′ RACE product, the S-SAP product at the S14 site and the PCR product including the transposed *LIb* copy are registered in DDBJ/EMBL/GenBnak as AB31837, AB31838 and AB31839, respectively.

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Site without insert CTAGTATTTAACACGAGAATACATTTTTAGTGTTCACTCTCATATTCTAATTTACAAATT
                    GCATTTATGATTATCAAAGTGGCAAATAGTAGTACAATATAATACACAGACTATATGCCT

                        (Site without insert sequence, 720bp omitted)

                    TATCTGTTGTCGTCATCATGCATGTATTCCTGTCTTCCATGCCTGCTGTTTTTAGAAGAA

Site without insert GAAGAAGAAGATGAGTATTGAAATCTTGAGAGTTGCAGACTAGCAGGAAATGGATATGAC
LIb at 14                                  ***********************************C**
                                          D1 primer at S14

Site without insert ATATATATAGATACATACATACATATACGTCATCCGTGTGATATATATAAGTGTATGTA-
LIb at 14           ************************************************************T

Site without insert ------------------------------------------------------------
LIb at 14           TTAGTGGCAAATGGCAATGGGGAGTAACAGGGCTCCGCTAGGTTTTCCTCTCTGCATCGC
                                          LIb-S14-A2 primer

Site without insert ------------------------------------------------------------
LIb at 14           GCCTCACCAGTGCGCCGCCTCCACTGCGTCGCCAGGCCACCGCGGTCCACGGCGCTGCGC

Site without insert ------------------------------------------------------------
LIb at 14           CGACGCTAACGCCGACGCCACCGCCTGTCGACCTCCGGCTTCTGCAGTTCTGGTTCGGCC
                                               LIb-S14-A1 primer

Site without insert ------------------------------------------------------------
LIb at 14           CTCGGCAGTCGACACCAGCCACGGCCCACAGCCACCAGTTTTGCAGAGAGCGGAAAAGCC

                        (LIb at 14 sequence, 5195bp omitted)

Site without insert ------------------------------------------------------------
LIb at 14           AGACGCTCGACCATCTTTTTCGGCGCTGCTTACTGGCGGAAGCCTGTTGGGATTCGGCCG
3'RACE product                 *T*****************************************

Site without insert ------------------------------------------------------------
LIb at 14           TACCTCCTCTCACTTTCCAGACATCCAACCATCTTCACATGCATAGTTGGATGAAGGCGG
3'RACE product      ************************************************C********

Site without insert ------------------------------------------------------------
LIb at 14           CATGCTCTTCTCAACAGAAGGATGGCTATAGTACTAACTGGTCGCTGATTTTTCCCTACA
3'RACE product      **********C******************G*****************************

Site without insert ------------------------------------------------------------
LIb at 14           TCCTTTGGAATTTGTGGAAGGCAAGAAACAGACTGGTGTTTGATAACAATATCACAGCCC
3'RACE product      ************************************************************

Site without insert ------------------------------------------------------------
LIb at 14           CCTCAGATATCCTCAACCGTAGTTTCATGGAATCAAGCGAAGCAAGATGTCTTCTGGCGA
3'RACE product      ****************************************A*******************

Site without insert ------------------------------------------------------------
LIb at 14           AACGAACCGGCCTCCAGACAGCCTTTCAAACGTGGGTGGTCTGGTCCCCTCCAGCGGCGG
3'RACE product      ************************************************************C*

Site without insert ------------------------------------------------------------
LIb at 14           GTTTCACTAAACTCAACTCAGATGGTGCTTGCAAATCTCACTCTCACCTTGCGAGCGCGG
3'RACE product      ************************************************************

Site without insert ------------------------------------------------------------
LIb at 14           GAGGACTGCTAAGGAATGAAAACGGGCTCTGGGTGGCAGGCTATACTTGCAACATTGGGA
3'RACE product      ************************************************************

Site without insert ------------------------------------------------------------
LIb at 14           CGGCTAACAGTTTCCTAGCCGAGTTATGGGGTCTTCGGGAAGGTCTTCTCCTTGCTAAAA
3'RACE product      ************************************************************
                          RT-D primer
Site without insert ------------------------------------------------------------
LIb at 14           ACCGAGGCTTCACCAAACTCATTGCTGAAACAGATTCTGAGGCTGTTGTTCAAGTGTTGC
3'RACE product      ************************************************************
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Figure 3. Continued.

polymorphic sites, S14, was selected for further analysis. Suppression PCR based on the host sequence of the S-SAP product using genomic DNA from Koukei14 (SZ) amplified the fragment at S14 without an *LIb* insert.

Aligning the sequences from the suppression PCR and S-SAP products allowed us to deduce the host sequence flanking the 5′ end of the *LIb* copy inserted at S14 (as in Figure 3). PCR amplified the region between the host sequences flanking the insert to produce a large fragment containing the *LIb* insert, and a short host fragment without the insert from the KB-4 callus genome. However, only a short fragment was amplified from the Koukei14 (SZ) genome. The sequence of *LIb* at the S14 insertion site was 6303 bp in length, and was highly homologous to the 3′ RACE product (850/855 bp match, Figure 3). A homology search of DNA and protein databases indicated that the inserted sequence consisted of the 5′ UTR of 566 bp, two ORFs of 1599 and 4101 bp with an overlap of 4 bp, a 3′ UTR of 30 bp, and the poly-adenylated tail of the LINE. There are characteristic cysteine motifs in ORFs 1 and 2, and the amino acid sequences of reverse transcriptase, endonuclease, and RNaseH are conserved among LINEs in plant species (Figure 4). The *LIb* insert at S14 is flanked by direct repeats of 19 bp (Figure 3). This type of duplication, called target-site duplication (TSD), occurs when non-LTR retrotransposons integrate into the host genome through target-primed reverse transcription (TPRT) (Luan *et al.*, 1993). TSD flanking the insert is decisive evidence for the transposition of *LIb* in KB-4 callus cells during tissue culture.

Suppression PCR was used to obtain full-length copies of *LIb* transposed into different sites of KB-4 callus using the *LIb*-S14-A1 and *LIb*-S14-A2 primers. These were designed based on the 5′ sequence of the 5′ UTR of the *LIb* copy transposed into the S14 site (Table 2 and Figure 3). PCR produced a few distinct bands from Koukei14 (SZ) genomic DNA, but many faint bands from KB-4 callus DNA (Figure 5). This outcome suggests that *LIb* copies sharing the 5′ UTR sequence with the S14 copy were present in a limited number in the original Koukei14 genome, although they were actively transposed in callus during tissue culture. Five products were cloned from suppression PCR using KB-4 DNA as template and sequenced. As was the S-SAP

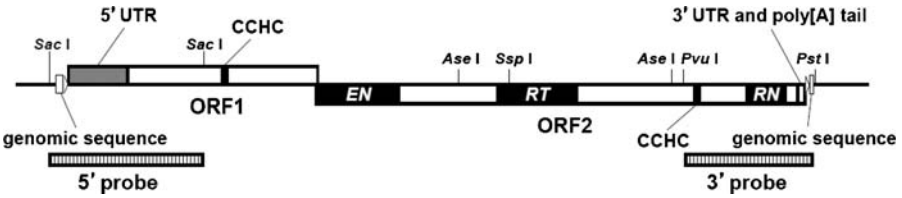

Figure 4. Schematic representation of the sweet potato LINE-type retrotransposon, *LIb*. The *LIb* copy transposed into the S14 site of the KB-4 callus genome was cloned with flanking host sequences into the pCR-XL-TOPO plasmid. The staggered rectangles indicate overlap between the two ORFs. The bold line at each end indicates the plasmid fragment. UTR, *EN*, *RT*, and *RN* stand for untranslated region, endonuclease, reverse transcriptase, and RNaseH, respectively. Open rectangles at each end are sweet potato genome fragments interrupted by *LIb* transposition. Filled boxes show CCHC, a possible zinc-finger motif in ORFs 1 and 2. Open triangles at each end show target-site duplications. Vertical lines with *Sac*I, *Ase*I, *Ssp*I, *Pvu*I, and *Pst*I are the restriction sites of these enzymes. The striped rectangles denote the corresponding positions of the 5′ and 3′ probes used for Southern and dot-blot analyses.

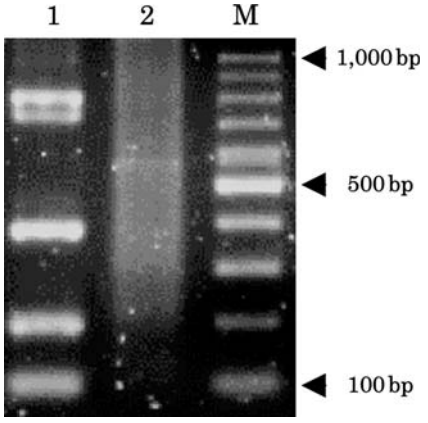

Figure 5. Suppression PCR of the genomic DNA of Koukei14 (SZ) (lane 1) and the KB-4 callus line (lane 2) based on the 5′ UTR sequence of the *LIb* copy transposed into the S14 site during tissue culture of KB-4.

product at S14, PCR between *LIb* and the flanking host genome sequences confirmed the transposition of *LIb* during tissue culture at all five sites, S10, S11, S12, S25 and S34 (data not shown). The host genome sequence flanking the 3′ end of the transposed copy at each of these five sites was determined by suppression PCR using A1 and A2 primers (Table 2).

PCR amplification between interrupted host sequences was used to isolate the fragment containing the transposed copy at these sites. A large fragment was amplified at two sites, S10 and S11, but not at the other three sites, S12, S25, and S34. The large fragments at the S10 and S11 sites were sequenced. The sequences of *LIb* from the S10 and S11 sites of 6365 and 6334 bp were 99.476% and 99.508% identical to that at S14, respectively. However, the 5′ ends of the UTRs varied (see Supplementary Material Figure 1 [SFigure 1]). Although the fragment containing a transposed copy was not amplified at S12, S25, or S34, PCR based on the 3′ sequence of *LIb* (L-D4 primer) and the flanking host sequence (the D1 primer at each site in Table 2) yielded the fragments at these sites. All fragments contained a 3′ sequence nearly identical to the comparable part of the *LIb* copy at S14. The flanking sequences matched those determined by suppression PCR of the 5′ host sequence. Alignment of the suppression PCR sequence with the flanking sequences, as for the S14 site in Figure 3, revealed TSDs of 14, 13, and 14 bp at the S12, S25, and S34 sites, respectively (SFigure 1). It is highly likely that a full-length *LIb* copy was integrated into these three sites. The KB-4 callus is most likely composed of various cell lines, each of which has a different history of recent *LIb* transpositions. Cell lines containing an insert at the S12, S25, or S34 sites were probably present in small numbers among cell populations of the KB-4 callus. This may make it difficult to amplify large fragments containing the *LIb* insert by PCR, as PCR amplification of DNA with inserts of over 6 kbp is extremely unlikely compared with the amplification of DNA with no insert, because PCR preferentially amplifies the smaller fragment.

Spontaneous transposition in plants

Because *LIb* is transcribed in the leaves and roots of Koukei14 (SZ) plants (Figure 1), spontaneous transposition might occur during normal plant growth and clonal propagation. This possibility was examined by comparing S-SAP patterns among various local lines of Koukei14. The clonal origins and propagation of these lines were verified by S-SAP based on the *Rtsp-1* sequence, which

exhibited an identical pattern for all these lines even though the S-SAP products were numerous (see SFigure 2). However, *LIb* S-SAP analysis of the same set of Koukei14 local lines with *Mse*I or *Alu*I as the restriction enzyme produced polymorphic products (Figure 6). Polymorphic bands that distinguished a line or group of lines were selected and sequenced: three sites, SCL3, SKO, and SNA, from the *Mse*I preparation, and one site from the *Alu*I preparation, SCL6. The flanking sequence at the 5′ end of the insert was deduced by suppression PCR using A1 and A2 primers at these sites (Table 2), and the entire insert was cloned, as was the insert from the cultured cell. PCR to amplify the region between the host sequences flanking the *LIb* insert at these sites verified the S-SAP results, i.e., the presence (+) or absence (−) of an insertion at each site in these lines (as shown in Figure 9). All inserts were highly homologous to the transposed copies in the cultured cells, but truncated at the 5′ end. Insert sizes ranged from 313 to 4161 bp, and TSDs of 10, 15, and 16 bp were found for the three inserts. However, for the 4161 bp insert at the SCL6 site, a genomic fragment of 12 bp was apparently deleted on the 5′ side of the insertion site (SFigure 3).

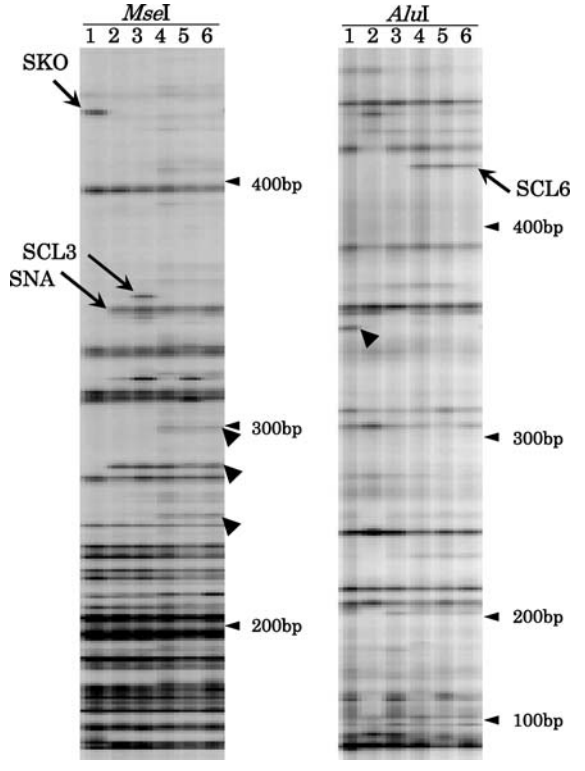

Figure 6. Sequence-specific amplification polymorphism (S-SAP) fluorographs of local lines of Koukei14 by the 3′ sequence of *LIb* using *Mse*I or *Alu*I as the restriction enzyme. Koukei14 (SZ) (lane 1), Naruto-kintoki (lane 2), CL3 (lane 3), CL6-1 (lane 4), CL6-2 (lane 5), and CL6-3 (lane 6). The S-SAP products denoted by arrows were cloned and the polymorphism observed on the fluorographs was confirmed by PCR. Arrowheads indicate other apparent polymorphic products.

Transposition during meristem culture

Plantlets with several nodes developed from surface-sterilized shoot apices of Koukei14 (SZ) placed on MS medium, and callus tissue formed in the basal region after 5 months in culture. Although the S-SAP patterns of four plantlets and their donor Koukei14 (SZ) plant were almost identical, at least two plantlets showed a unique single band (Figure 7). After the product specific to plantlet 4 was cloned (SY30), the region between *LIb* and the adjacent host sequence was PCR amplified. Results showed that the junction of *LIb* and the host genome

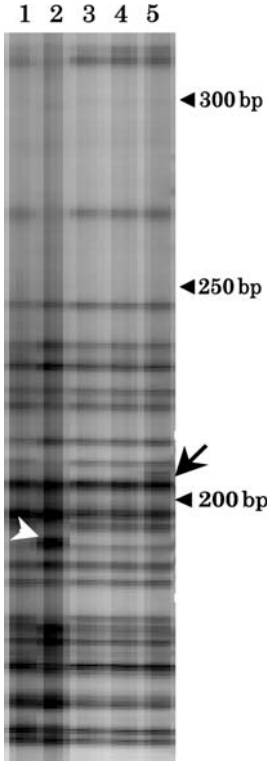

Figure 7. Sequence-specific amplification polymorphism (S-SAP) fluorographs based on the *LIb* sequence for four plantlets (lanes 2–5) developed from meristem culture of Koukei14 (SZ) plants (lane 1). Polymorphic product of 210 bp in lane 5 indicated by an arrow was cloned and sequenced. An arrowhead indicates another apparent polymorphic product.

was present only in plantlet 4; it was absent in the donor Koukei14 plant and other plantlets (data not shown).

After the host genome sequence flanking the 5′ end was inferred by suppression PCR using A1 and A2 primers at the SY30 site (Table 2), the transposed copy was isolated by PCR amplification of the region between the sequences flanking the copy. The copy was found to be largely truncated from the 5′ end to 540 bp, and a genomic fragment of 23 bp was deleted on the 5′ side of the insertion site (SFigure 3). This was also the case for the SCL6 insert described above. The same PCR method using genomic DNAs from Koukei14 (SZ) and four plantlets as templates confirmed that the *LIb* insertion at SY30 was present only in plantlet 4 (data not shown). These results demonstrate that the transposition of *LIb* into the SY30 site occurred as an independent event in plantlet 4 during meristem culture. Although the mechanism of deletion at insertion sites is unclear, deletions or inversions upon insertion are occasionally observed in other LINE elements (Moran *et al*., 1996; Symer *et al*., 2002).

Southern blotting analysis

The S-SAP method can identify relatively short (up to 800 bp) fragments defined by the nearest restriction site to the 3′ sequence of *LIb* with extremely high resolution. However, Southern blot analysis can visualize all sizes of restriction fragments containing the *LIb* sequence, but with considerably reduced resolution. Southern analysis of Koukei14 and its local line, Naruto-kintoki, showed similar results to the S-SAP method: a number of identical bands with a few polymorphisms between them (Figures 6 and 8A). All newly transposed copies in plants determined in this study were truncated at the 5′ end. However, polymorphisms were observed when the 5′ sequence of *LIb* was examined with a Southern probe. This indicated full-length *LIb* insertion by transposition after divergence of these two lines. Southern analysis of callus tissue produced an increased number of hybridizing bands from the callus lines (Figure 8B). A substantial increase in bands indicates that *LIb* transposition has taken place continuously in different cells of the callus line.

Copy number of LIb

Reverse transcription and integration of a LINE into the host genome occurs in a series of processes called TPRT (Luan *et al*., 1993). Products not fully reverse-transcribed from the 3′ end of LINE RNA frequently complete their integration into the host genome, resulting in 5′ truncated copies (Ostertag and Kazazian, 2001). Dot-blotting analyses with the 5′ probe estimates the relative content of full-length *LIb* DNA present in the sweet potato genome. This estimate from Koukei14 was 0.0071% (SD 0.000077%), which would be equivalent to 108 copies in the hexaploid genome assuming a haploid genome size of 1597 Mb (Arumuganathan and Earle, 1991) and a full-length *LIb* of 6300 bp. On the other hand, analysis using a 3′ probe provides a relative estimate of all *LIb* copies, including those truncated at the 5′ end. This was calculated to be 0.0095% (SD 0.00017%), and the value was significantly ($P < 1\%$ by *t*-statistics) larger than the estimate made with the 5′ probe.

Because hybridization conditions, including the effective probe lengths (1153 and 1040 bp for the 5′ and 3′ probes, respectively), were virtually equivalent in the two analyses, the difference of 0.0024% accounts for the 5′-truncated *LIb* copies, which range from those narrowly undetected by the 5′ probe to those barely detected by the 3′ probe, i.e., from a little over 5200 bp to around 1000 bp in length. If the average size of these 5′-truncated copies were 3000 bp, this number would be around 73 per genome; all copies detected by the 3′ probe would sum to 181 per genome. Because *LIb* copies lacking the 5′ UTR region are unable to transcribe by themselves, copies truncated at the 5′ end are likely to have originated from full-length transcripts. Integration of partially reverse-transcribed products into the host genome appears a common phenomenon during the spontaneous transposition of *LIb* in wild plants.

Spontaneous LIb *transposition among local Koukei14 lines*

Koukei14 has been a popular cultivar in the western part of Japan for more than 50 years. Despite vegetative propagation, several off-types

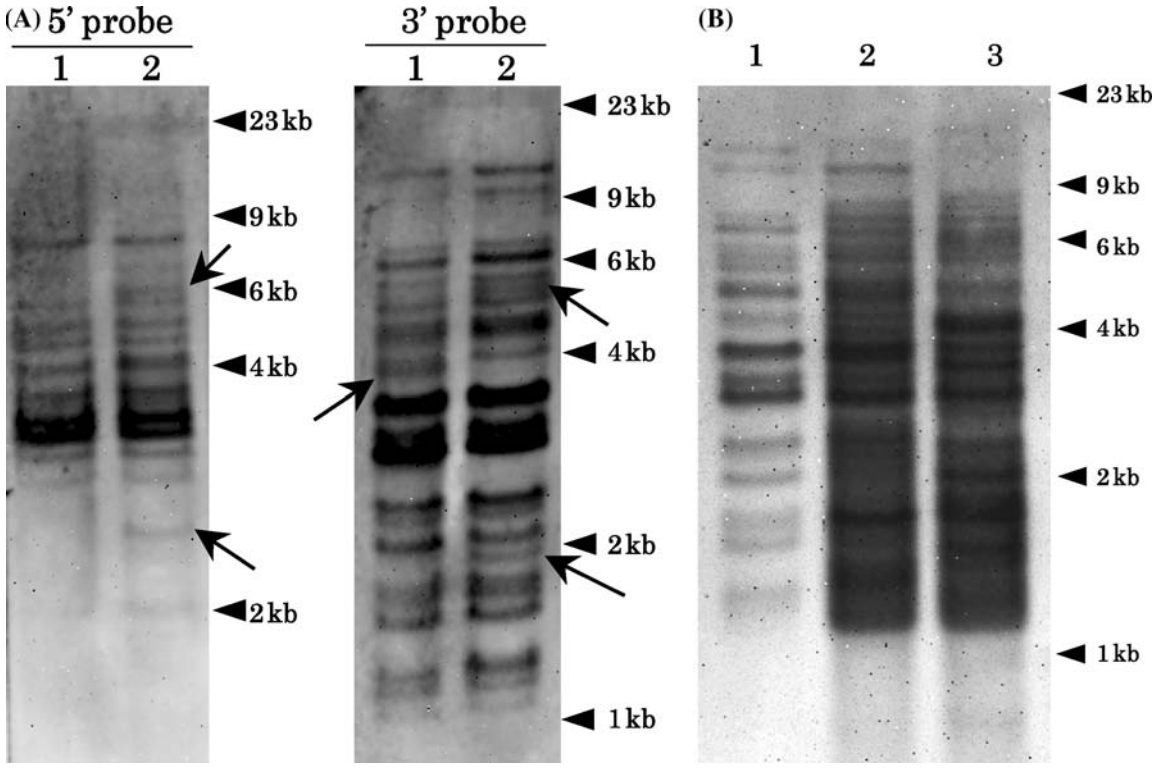

Figure 8. (A) Southern blot analysis of Koukei14 (SZ) and its local line, Naruto-kintoki CL3, using the sequence of the transposed *LIb* copy as probe. The 5′ probe contained an entire 5′ UTR (566 bp) and the adjacent ORF1 portion (587 bp), whereas the 3′ probe consisted of the 3′ portion of ORF2 (999 bp), the short 3′ UTR (30 bp), and a poly-adenylated tail (11 bp) (see Figure 4). Arrows indicate possible polymorphic products. (B) Southern blot analysis of Koukei14 (SZ) (lane 1), and the two callus lines, KB-4 (lane 2) and KB-4R (lane 3) derived from it, using the 3′ probe from the transposed *LIb* copy.

have been discovered to which local names have been given. These local lines may be divided into two groups based on storage root skin color (the original is brownish and the mutant reddish). Such lines include Tosabeni and its descendants. Other than root characteristics, there are few morphological differences that effectively distinguish these lines. Indeed, no S-SAP patterns produced by the LTR retrotransposon *Rtsp-1* were discernible among these lines, suggesting that mutational differentiation is minimal. The lineages of the local Koukei14 lines have been deduced on the basis of their discovery (Figure 9). In this study, we found four spontaneous transpositions of *LIb* among these lines. Once inserted, LINEs do not excise. Therefore, if a group of lines share an *LIb* insertion at a particular site, these lines must be derived from the same common ancestral line in which that insertion occurred. The occurrence of the four *LIb* transpositions inherited among Koukei14 lines was examined by PCR insertion analysis (see Figure 4). The SNA insert was originally discovered as common to Naruto-kintoki and its clonal selections, CL3, CL6-1, CL6-2, and CL6-3 (Figure 6). However, PCR analysis clearly showed the presence of this insert in Tosabeni, but not in Koukei14. Therefore, the *LIb* transposition must have occurred in Tosabeni or its clonal ancestor. The lineage relationships determined by *LIb* insertions were entirely consistent with the lineage tree constructed from the record of their discovery (Figure 9). This provides convincing evidence that spontaneous *LIb* transpositions occurred during the diversification of these lines, and that the inserted *LIb* copies were stably inherited.

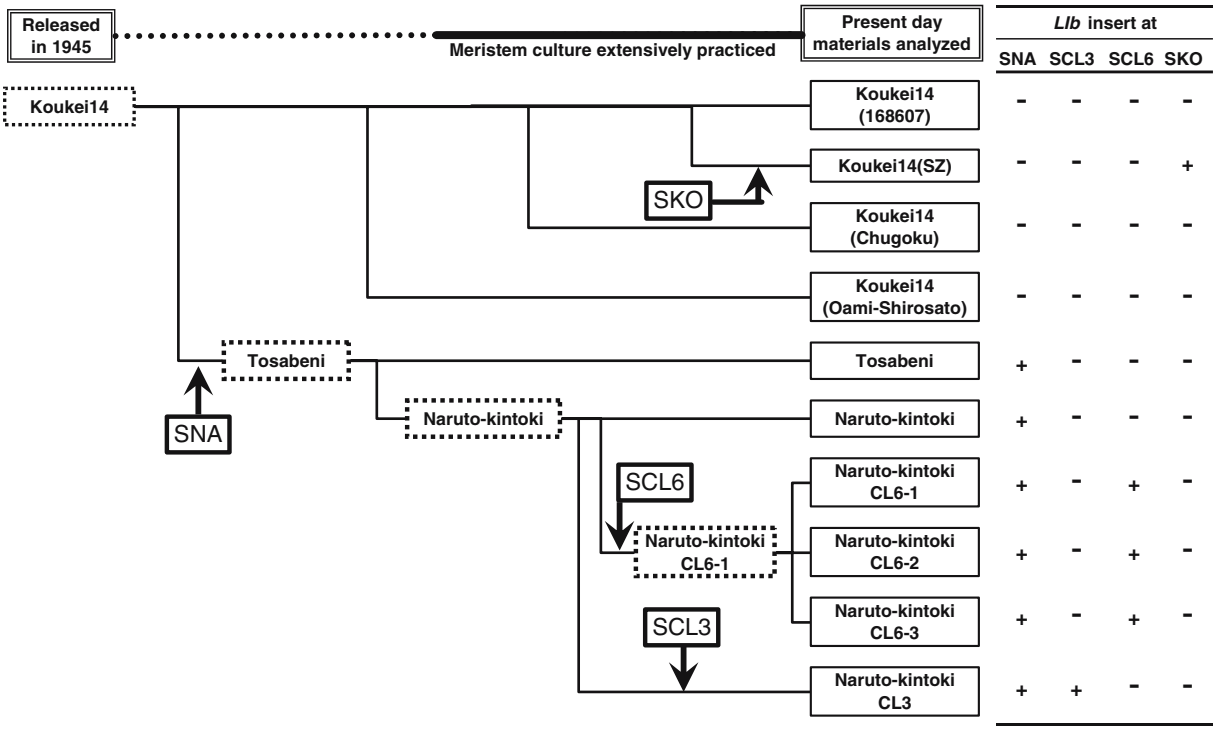

Figure 9. Lineage of local Koukei14 lines based on records of their discovery and the possible occurrence of *LIb* spontaneous transpositions deduced from PCR confirmation of *LIb* insert in the SNA, SCL3, SCL6 and SKO sites of the Koukei14 lines. Arrows indicate points in the Koukei14 lineage at which *LIb* transposed into the SKO, SCL3, SCL6, and SNA sites. The table on the right side of the chart indicates the presence (+) or absence (–) of the *LIb* insert in the SNA, SCL3, SCL6 and SKO sites of the Koukei14 lines as shown in the boxes in the lineage tree. The *LIb* insert at these sites was confirmed by PCR amplification of the fragments flanking the insert (see SFigure 4). The transposition points were unambiguously determined as the only members of Tosabeni and Naruto-kintoki CL6-1 descendants in the lineage tree that shared insertions at SNA and SCL6, respectively. Insertions at SCL3 and SKO were unique to Naruto-kintoki CL3 and Koukei14 (SZ), respectively.

Discussion

Characteristics of an active LIb *member in the Koukei14 genome*

We isolated a LINE-type retrotransposon, *LIb*, which was actively transposed in sweet potato callus derived from the cultivar, Koukei14, and observed spontaneous transposition among local lines of Koukei14. We first identified an apparent full-length *LIb* copy transposed into the S14 site of the KB-4 callus genome during culture. Suppression PCR analysis based on the 5′ UTR sequence of the S14 copy revealed that copies sharing the 5′ UTR sequence with the S14 copy were present in a limited number in the original Koukei14 genome, but had increased dramatically during culture (Figure 5). The sequences of these apparent full-length copies, as far as they could be ascertained, were almost identical to that at S14. As such, their 3′ sequences were very similar to those of the transposed copies in the Koukei14 lines and mericlone plantlets, as well as to the transcripts (3′ RACE products) in the KB-4 callus. These results suggest that (1) active *LIb* copies in the original Koukei14 genome belong to a single LINE family represented by the S14 copy, (2) copies containing the 5′ UTR of the S14 copy or an additional upstream sequence are quite actively transposed in the callus, and (3) active members with long functional 5′ UTRs are present only in limited numbers in the Koukei14 genome. Newly transposed copies in KB-4 calluses with untruncated 5′ UTRs may retain their transcriptional capacity. If this is the case, repeated transpositions after the addition of new competent copies might have caused an exponential increase in the numbers of copies in the callus genome.

Because KB-4 and KB-4R have been maintained as calluses for over four years, they should have become a mixture of various cell lines, each possessing different new inserts. This possible mosaic nature of the callus lines may partly account for the detection of a large increase in the S-SAP or Southern blotting bands of the callus lines.

LIb *transposition induced meristem culture*

Meristem culture of Koukei14 allowed the transposition of *LIb* in plantlets developed from it. To date, only five retrotransposon families have been shown to be transpositionally competent in plant species: *Tnt1*, *Tto1*, *Tos17*, and *Rtsp-1* of the LTR retrotransposons, and *Karma* of the non-LTR transposons (Grandbastien *et al*., 1989; Hirochika, 1993; Hirochika *et al*., 1996; Komatsu *et al*., 2003; Tahara *et al*., 2004). However, transposition of these active retrotransposons has only been shown in cultured tissue or in plants regenerated from tissue culture. In the case of sweet potato meristem cultures, the apical tips of the meristems are unlikely to pass through an undifferentiated callus stage before plantlets develop. Therefore, *LIb* transposition by meristem culture is the first experimental demonstration of the *in planta* transposition of retrotransposons induced without differentiation experimentally imposed on plant cells.

Transposition activity in meristem tissue

Due to its susceptibility to sweet potato feathery mottle virus, Koukei14 was propagated exclusively in meristem culture after the late 1980s to produce virus-free stock for growers (Shimonishi *et al*., 2001). One *LIb* insertion, SNA, is present in Tosabeni, as in Figure 9, which was discovered before meristem culture became the practice. However, the other three insertions appear to have occurred in lines grown in meristem culture. This entails to two important conclusions: (1) *LIb* in the Koukei14 genome is able to transpose spontaneously in wild-type plants, and (2) meristem culture may promote this transposition.

The new insertion of *LIb* at the SY30 site was detected in all leaves of the mericlone plantlets, as well as in callus tissue formed at the bottom of the plantlets based on the PCR to amplify the fragments flanking the *LIb* insert (data not shown). Other recent *LIb* insertions in the Koukei14 lines are also persistent over vegetative generations (see Figure 9). These results suggest that cells with any of these *LIb* insertions are the only cell type to exist in an entire plant. They are not present in a mosaicism with cells lacking the insertion. This implies that the leaves and calluses of mericlone plantlets originated from a single initial cell in which new insertions of *LIb* had occurred. After insertion, this initial cell continuously reproduced itself, giving rise to differentiating progeny cells. The shoot tip meristem is composed of the central zone at the apex, the peripheral zone towards the flanks, and the rib zone beneath it (Fletcher, 2002). The initial cell, also called a stem cell, occurs in the central zone, and its multiplication facilitates the formation of leaves and other organs, and is involved in shoot growth. In the case of meristem culture, divided cells that move toward the peripheral zone are differentiated into leaves and other organs as an intact apex. However, those moving downwards to the rib zone may initially form calluses instead of shoots. It has been hypothesized that stem cells change over time, and that there are no permanent stem cells (Stewart and Dermen, 1970; Zagórska-Marek and Turzańska, 2000). Our results contradict this hypothesis, clearly indicating cell lineages arising from single permanent stem cells. As indicated above, spontaneous insertion at the SNA site must have taken place in a single permanent stem cell in the meristem of field-grown wild-type plants of Tosabeni or its clonal ancestor. When dissected shoot apices of sweet potato are placed on artificial medium, meristem activity may be temporarily suspended, but it will resume when a single stem cell initiates activity under the influence of artificial environmental conditions. Although the mechanism is unknown, dramatic changes in growth conditions induced with artificial culture supplements may increase the likelihood of *LIb* transposition in the founder stem cells of the meristem.

New insertions of a retrotransposon are inherited and detected only when cells with the insertion form the entire tissue of an individual plant or at least form a mosaic. For vegetative propagation to fulfill this condition, the transposition must take place in stem cells in the meristem, and the stem cells must remain permanent during plant development, as described in the aforementioned mericlone plantlet case. In the case of sexual propagation, if

germ cells are formed from stem cells with a recent transposition during reproductive organ differentiation, or transposition occurs during germ cell formation (as with a human L1 element; Ostertag and Kazazian, 2001) or the fertilization process, progeny plants will be entirely composed of cells with the insertion. In either case, retrotransposons must transpose in these founder cells.

Transposition in the stem cells of the meristem is readily inherited or fixed in descendant plants, not only through sexual propagation, but also through vegetative propagation. This type of retrotransposon activity must have occurred in many cases of retrotransposition, and changes in the plant genome or genetic system due to retrotransposition become genetically inherited characteristics. Because *LIb* is activated in the stem cells of sweet potato in meristem culture, and has apparently left recent spontaneous transposition products in the sweet potato genome, this LINE-type element can be a useful tool in studying the transpositional activities of plant LINE-type elements, their effects on host genetic systems and organization, and their control by host plants.

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