Majin: A Miniature DNA Element Associated with the Genomes of Pome Fruit Trees

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Abstract. Sequencing of the sequence tagged site-DNA marker fragments linked to the fruit skin-color gene in apple revealed two fragments containing insertions that are associated with yellow skin color, and are alleles of the red skin gene (Rf). One fragment resulted from the insertion of a 76-bp inverted repeat, whereas the 163 bp in the other fragment was characterized as a mobile element because of the presence of target site duplication. A database search found this latter element in the 5' flanking regions or intron of six apple genes. DNA blot analysis revealed that the element is highly reiterated, with a copy number of between 5000–6000 in the genome of Maloideae (pome) fruit trees. We named this element *Majin*. Inter-transposon amplified polymorphism using primers from this element resulted in polymorphic bands among apple cultivars.

Through a combination of mutant analysis, characterization of DNA polymorphisms within populations, and computer-based surveys, some highly repetitive transposable elements have been found in many eukaryotic genomes. Transposable elements have been divided into two main classes according to their transposition intermediate (Flavell et al., 1994). Class 1 elements include retroposons with a long terminal repeat (LTR) such as Ty1/Copia-like and Ty3/Gypsy-like elements, as well as no-LTR retroposons. Plant genomes contain high copy numbers of these retroelements (Mao et al., 2000). The Class 2 elements transpose via a DNA intermediate and usually have terminal inverted repeats. They consist of autonomous and nonautonomous members such as AC/DS and Mutator (Federoff, 1989). Miniature inverted-repeat transposable elements (MITEs) have similarities in both terminal inverted-repeats (TIRs) sequence and target site duplication (TSD) to those of autonomous class 2 transposons. Therefore, it has been hypothesized that MITEs represent nonautonomous forms of DNA-based transposable elements (Wessler, 1998). The abundance of MITEs in plant genomes has been reported (Bureau and Wessler, 1992, 1994; Casacuberta et al., 1998; Mao et al., 2000;

Pozueta-Romero et al., 1996). More recently, a putative autonomous element of *tourist*-like MITEs in the maize genome was discovered (Zhang et al., 2001).

Other miniature elements that are not in accordance with the characters of MITEs have also been found (Charrier et al., 1999; Song et al., 1998). These elements appear to represent a new class of miniature transposable elements. Through sequencing of the sequence tagged site-DNA (STS-DNA) marker fragments linked to apple fruit skin color, we have identified a highly reiterated miniature element in the genome of apple and related plant species. We report on the characteristics of this sequence that we named *Majin* (*Malus* element identified by Jin).

Materials and Methods

Plant material. The following plants were used in this study: cultivated apple (*Malus ×domestica* Borkh. cvs. Fuji, Golden Delicious, Jonathan, Narihokoh, Hirosaki Fuji, Indo), wild apple species (*M. prunifolia* Rehd., *M. hupehensis* Rehd., *M. pumila* Mill.), pear (*Pyrus communis* L.), Japanese pear (*Pyrus pyrifolia* Nakai), quince (*Chaenomeles lagenaria* Koidz., *Pseudocdonia sinensis* Schneid., *Cydonia oblonga* Mill.), rowan (*Sorbus commixta* Hedl.), spiraea (*Spiraea thunbergii* Sieb., *Spiraea cantoniensis* Lour.), and cherry (*Prunus avium* L.). Young expanding leaves of these plants, obtained at the experimental farms of Hirosaki Univ. (Japan), were used for DNA extraction.

DNA extraction. Genomic DNA was isolated from leaves by the method of Varada-

rajan and Prakash (1991) and purified by the ethidium bromide-CsCl method of Sambrook et al. (1989).

Sequencing. By using the PCR primers described by Cheng et al. (1996), three DNA markers (A1, a1, and a2) linked to skin-color were amplified from 'Jonathan', 'Narihokoh', and 'Golden Delicious', respectively. 'Jonathan' and 'Golden Delicious' are chance seedlings and 'Narihokoh' is a cross between 'Golden Delicious' and 'Fuji'. Each amplified fragment was subsequently cloned into the pBluescript® IISK(-) phagemid vector (Stratagene, La Jolla, Calif.). Both strands were sequenced using an automated sequencer (LI-COR, Lincoln, Neb.) with IRD-labeled M13 forward and reverse primers.

Slot and DNA gel blot hybridization. *Majin* DNA (153 bp) was amplified by PCR from the cloned a2 fragment using the primers A (5'-GGGAACCTTTAACGAAAAGAAGC-3') and B (5'-GGAAACTAGTGA AAATGGCTTG-3'). Serial dilutions corresponding to 10³–10⁵ copies per genome were slot blotted, along with 50 ng of genomic DNA extracted from young leaves of 'Golden Delicious' apple, onto a membrane (Hybond N+, Amersham). In this calibration for copy number, 750 Mbp per 1C was used for the DNA content of apple (Arumuganathan and Earle, 1991). For gel blot analyses, one µg of genomic DNA extracted from young leaves was digested with *Hind*III, separated by agarose gel electrophoresis and transferred to the membrane. For both blots, *Majin* probe DNA was labeled with ³²P-dCTP using the Prime-It II random primer labeling Kit (Stratagene). Membranes were hybridized with the labeled probe at 65 °C for 15 h in hybridization buffer (10 mM tris-HCl, pH 7.5, 1 mM EDTA, 6×SSC, 5×Denhardt's solution, 0.2 mg·mL⁻¹ salmon sperm DNA, 20 mM sodium phosphate buffer, and 1% SDS). The membranes were washed with 2×SSC/0.1% (w/v) SDS at room temperature for 30 min and 0.2×SSC/0.1% (w/v) SDS at 65 °C twice for 15 min and then autoradiographed at -80 °C with Kodak X-ray film and an intensifying screen. Scanning densitometry of slot blot autoradiograms was performed using a densitograph of ATTO (AE-6920M-05, Tokyo).

Inter-transposon amplified polymorphism (ITAP). Primers A or B as inward primers and C or D (complementary sequences of A and B, respectively) as outward primers were used for ITAP PCR. A total volume of 25 µL reaction mixture contained 100 ng genomic DNA, 1×PCR buffer (Applied Biosystems, Foster City, Calif.), 2.5 mM MgCl₂, 0.5 µM primer, 200 µM dNTP, and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR was carried out in a TaKaRa PCR Thermal Cycler MP (Takara Shuzo, Tokyo). The PCR cycling parameters consisted of a 10-min denaturation at 95 °C, followed by 40 cycles of 1 min at 94 °C, 2 min at 52 °C, 3 min at 72 °C, followed by a final 9-min extension at 72 °C. Amplified products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. DNA size markers (λDNA/Styl and φX174/HaeIII digests) were purchased from Nippon Gene (Tokyo).

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Results

Detection of *Majin*. By using the universal primers designed by Cheng et al. (1996) and the resulting A1, a1, and a2 markers, we identified six genotypes arising from combinations of the A1, a1, and a2 markers in selected apple cultivars. The marker genotypes correlated with the skin-color phenotypes, with marker A1 co-segregating with red skin color and a1 and a2 associated with yellow skin color (Cheng et al., 1996). To investigate the molecular basis of this polymorphism, we sequenced these fragments. Each DNA marker fragment was cloned from its homozygous cultivar (A1 from 'Jonathan', a1 from 'Narihokoh', and a2 from 'Golden Delicious'). The sequence information indicated that the a1 (GenBank Accession No. AB073703) and a2 (GenBank Accession No. AB073704) fragments were virtually identical to A1 (GenBank Accession No. AB073702) except for their respective insertions (Fig. 1A). Sequence data revealed that the 76 bp a1 insertion was an inverted repeat, while the 163 bp insertion in a2 contained a duplication of a 10-bp target site (Fig. 1B). When the a2 insert was used in a computer-based sequence similarity search, six homologous sequences in the 5' noncoding regions or intron of six previously sequenced apple genes were identi-

fied (Fig. 2). Although the sequence in *MdPI* had a 28-bp deletion, the remaining region showed high homology to the a2 insertion. Because of the lack of information about the insert/deletion alleles of these six homologous loci, we are unable to determine whether the flanking sequences of the homologues are duplication products. However, their borders were composed mainly of AT. We named this 153 bp of a2 insertion sequence *Majin* (*Malus* element identified by Jin).

Copy number and distribution of *Majin*. The approximate copy number of *Majin* in apple genomic DNA was determined by slot blot hybridization (Fig. 3). Using high stringency washes, densitometer measurements showed ≈ 6000 copies per haploid genome. *Majin* was also used as a probe for DNA gel blots to determine the distribution of this element in other plant species. A positive signal was observed in the genomes of domestic apple cultivars (Fuji), wild apple species, pears, quince species, and rowan. No signal was detected in rice (*Oryza sativa*), soybean [*Glycine max* (L.) Merrill], spiraea, or cherry (Fig. 4). Although similar signal intensities were observed among apples, pears, and two quinces, about half of and twice the intensity were obtained from *Chaenomeles lagenaria*, while *Sorbus commixta*, respectively.

ITAP. Because MITEs are highly repetitive and present in both orientations in the genome, a single primer (e.g., A or B) can amplify PCR products from elements sufficiently close to one another and in the correct orientation (Kalendar et al., 1999). The dispersion, ubiquity and prevalence of *Majin* in the genomes of the pome fruits provide an excellent opportunity for the development of marker systems. To investigate this possibility, inward-facing PCR primers for the 5' and 3' ends (Fig. 2, primers A and B) were used to amplify DNA from five apple cultivars to determine whether these ITAP markers were sufficiently polymorphic. The ITAP bands were generated by amplification using either primer A or B (Fig. 5). Although the amplified patterns were identical between a cultivar and its sport (lanes 1 and 2, Fig. 5), each cultivar produced a different banding pattern. Two outward-facing primers corresponding to respective complementary sequences of primer A and B (Fig. 2, primer C and D) designed for both ends of *Majin* also produced ITAP (data not shown). PCR amplification using both outward primers or outward and inward primers increased the number of amplified bands (data not shown). However, some combinations resulted in unsatisfactory resolution of the bands for determination of polymorphism because specific bands were amplified excessively.

Fig. 1. (A) Schematic representation of the characterization of STS-DNA markers linked to apple fruit skin color. Marker A1 comprises a 1117-bp sequence. Markers a1 (1193 bp) and a2 (1280 bp) each have insertions in A1 at the positions indicated. Size of each restriction fragment and the restriction site are indicated. H = *HindIII*, E = *EcoRI*, B = *BamHI*. (B) Sequences flanking the a2 insertion site.

Fig. 2. Sequence alignment of *Majin* members of a2 and six apple genes from GenBank. The 10 bases at each end indicate the flanking regions of each *Majin*. Conserved nucleotides are indicated by white letters on a black background. Primers used in this study are shown. GenBank accession numbers and coordinates are: *MdRfa2*, AB073704, 869-1041; *MdACS1*, U89156, 596-768; *Mald1*, AF020542, 228-400; *MdPI*, AJ291491, 1515-1657; *MsPI*, AF350245, 228-400; *ABG1*, A84614, 1307-1134; *YPR10°C*, AY026910, 159-1.

Discussion

MITEs are characterized by their small size (between 110 and 500 bp), their A+T richness (>60%), the absence of coding regions, strong target site preference for a 2- to 4-bp sequence (TA, TAA, TTAA), the presence of TIRs, and high reiteration (Bureau et al., 1996; Wessler et al., 1995). Although the *Majin* of *MdRfa2* has a 10-bp target site duplication, it is not clear whether the border sequences of other *Majin* elements also contain the duplication. Furthermore, the TIRs observed in MITEs are imperfect in the *Majin*. From these characteristics, *Majin* elements closely resemble, if anything, *Snap*, *Crackle*, and *Pop* in rice (Song et al., 1998). The classification of the *Majin* element requires further investigation.

A positive *Majin* hybridization signal was obtained on DNA gel blots of *Malus*, pears, quinces, and rowan. All of these genera belong to the Maloideae, one of the four subfamilies of the Rosaceae (Chapbell et al., 1995). No signal was detected in cherry and two spiraea samples, which belong in the Amygdaloideae and Spireoideae, respectively. These results suggest that *Majin* is a sequence specific to the

Maloideae. The Maloideae, which include several commercially important fruit crops (Westwood, 1993), are thought to have originated as allopolyploids from Amygdaloideae and Spiraeoideae ancestors (Sax, 1931; Stebbins, 1950; Challice and Kovanda, 1981; Phipps et al., 1991). Thus, there is particular interest in whether *Majin* is present in subfamilies suspected to be ancestors of the Maloideae.

Although a high reiteration of *Majin* was revealed in the Maloideae, a computer search found only six apple genes harboring *Majin*. However, there are very few deposited sequences from the Maloideae, especially from flanking regions where most MITEs have been identified (Wessler et al., 1995). Many additional *Majin* elements will be reported as sequence records increase. Pome fruit trees frequently produce bud sport mutations that have very interesting morphological traits (Fisher, 1970; Lapins, 1969), including variations in intensity of fruit color (Bishop, 1959). Accessing these mutated loci using the highly reiterated *Majin* elements will contribute valuable information which can be used for both fundamental and applied genetic problems.

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Fig. 3. Copy number of *Majin* in the apple genome. Genomic DNA from the apple cultivar 'Golden Delicious' and *Majin* (*MdRfa2*, PCR product), representing from 10^3 to 10^5 copies relative to the amount of the genome, were diluted serially and transferred to a nylon membrane, then probed with *Majin*.

Fig. 4. Species distribution of *Majin*. One μ g of *Hind*III-digested genomic DNA was electrophoresed in a 0.8% agarose gel, transferred to a nylon membrane, and probed with *Majin*. (A) Lane 1 = *M. domestica* cv. Fuji, 2 = *M. pumila*, 3 = *M. prunifolia*, 4 = *Oryza sativa* cv. Nipponbare, 5 = *Glycine max* cv. Williams. (B) 1 = *Pyrus communis*, 2 = *Pyrus pyrifolia*, 3 = *Pseudocarya sinensis*, 4 = *Chaenomeles lagenaria*, 5 = *Cydonia oblonga*, 6 = *Sorbus commits*, 7 = *Spiraea thunbergii*, 8 = *Spiraea cantoniensis*. Data not presented for *M. hupehensis*, *Pyrus communis* (positive signal), and *Prunus avium* (negative signal). Marker DNA fragment sizes are indicated to the left of the gel in kbp.

Fig. 5. Banding patterns generated by ITAP from apple cultivars. An agarose gel showing PCR products generated with primer A or B (Fig. 2). Lane M1 = DNA size marker λ DNA/*StyI*, 1 = Fuji, 2 = Hirosaki Fuji (a sport of Fuji), 3 = Golden Delicious, 4 = Jonathan, 5 = Indo, 6 = negative control (rice DNA), M2 = DNA size marker ϕ X174/*Hae* III.

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